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<http://dx.doi.org/doi:10.21954/ou.ro.0001022a>

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Resistance to powdery mildew in wheat mutants ^{UNRESTRICTED}

Vivek Duggal

A thesis submitted to the Open University for the
Degree of Master of Philosophy

December 1998

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Acknowledgement

I am indebted to Prof. Graham Jellis, my Director of studies, for his constant support and advice in all matters. I am also highly thankful to Bill Hollins, Rebecca Stratford and Robert Shields for their regular guidance and constructive criticism of my work. I would also like to thank Graden Smith and Alan Taylor for their support during my pathological work.

I duly acknowledge the help and encouragement of all my friends especially John, Richard, Dan, Andy, Chris, D.P, Mel, Kathie, Helen *et al.* I would also like to thank everyone at PBI especially in the Plant Tech. for always making me feel at home.

I am grateful to PBI, Cambridge for funding my studies and Tony Worland for Hobbit Sib mutants.

Last but not the least I would like to thank my parents for their unfailing support and encouragement.

Abstract

Suppressors of resistance genes have been reported to exist in hexaploid wheat signifying that the expression of resistance depends upon the genetic background of the plant. In 1982, 'Hobbit sib' hexaploid wheat, was irradiated with fast neutrons and a series of deletion mutants with increased adult plant resistance to rusts and mildew was identified. In the present study this deletion series was evaluated for mildew resistance at various growth stages to identify and characterise resistance. Two mutants, I3-54 and I3-48 were found to be significantly more resistant than the wild type at various adult and seedling growth stages with the resistance appearing to be fully activated at the seedling third leaf stage.

The third leaves of these two mutants and 'Hobbit sib' were used for histological studies, to determine the rate of fungal hyphae development. There were no differences in the rate of spore germination and hyphal development during the first 12 hours after inoculation (hai) but by 18 and 24 hai both the mutants had on average a slower rate of hyphal development. This demonstrates that resistance is initially expressed at the pre-haustorial or hyphal penetration stage in the two mutants. By 72 hai, there were significant differences between the proportion of germinating spores forming haustoria in the three genotypes. Large differences in the colony sizes for one mutant compared to Hobbit sib were detected indicating the possible role of post-haustorial resistance also.

To study the association of various pathogenesis related (PR) proteins with this resistance, RNA from the uninfected third leaf of the mutants and 'Hobbit sib', was extracted and hybridised with cDNA probes of two PR proteins; PR1 and thaumatin like (TL). The expected sized mRNA transcript bands were detected for PR-1 and TL proteins in the two resistant mutants only. The results suggested that the constitutive over-expression of PR-1 and TL might be contributing to the resistance of I3-48 and I3-54.

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Hexaploid wheat (*Triticum aestivum*), the foremost cereal crop of world, is attacked by a vast array of pathogens. Particularly important among these are the foliar diseases, especially powdery mildew (*Erysiphe graminis*) and rusts (*Puccinia* spp.), which decrease the active photosynthetic area of the plant, resulting in substantial yield losses (McIntosh, 1998). Powdery mildew is an important disease in the cool and humid conditions of Western Europe. Yield losses of up to 40 % have been reported in epidemic years (Agrios, 1988). The most economical and environmentally safe method for controlling powdery mildew is to develop resistant cultivars. To date, twenty-four mildew resistance gene loci have been identified and these genes are frequently being used in various breeding programmes (Huang *et al.*, 1997). At the same time new virulent races of *E. graminis* are evolving due to sexual and asexual recombinations, mutations and gene flow (Burdon and Silk, 1997).

1.1 Evolution of wheat and its significance

Wheat was one of the earliest plants cultivated by man and played an integral part in the development of most of the major civilisations around the world. Its centre of origin lies in that part of Middle East described as the Fertile Crescent. This is an area covering the Mediterranean coastal plains of northern Israel and the Lebanon, the fertile regions of Syria and the Tigris and Euphrates valleys in the present day Iraq, where wild relatives of present day cultivated wheat are still prevalent.

The first steps in the evolution of wheat probably took place at least 10000 years ago with a natural hybridisation between the wild grasses, *Aegilops* spp. and *T. urartu* (diploid progenitors of hexaploid wheat, 14 chromosomes). This gave rise to a wild form of Emmer wheat (tetraploid wheat, 28 chromosomes) *T. dicoccoides*, which

has fragile ears which shatter into individual spikelets on ripening. Each grain in this type of wheat is protected by a very tough and tightly adhering covering to the husk. Neither of these properties is useful to the farmer and thus this might have led to another conscious or unconscious selected form of cultivated Emmer wheat, *T. dicoccum*, which is less fragile and easier to thresh than its wild relative.

This form of Emmer wheat was widely cultivated during the Neolithic period and gradually spread throughout central Europe to reach the British Isles before 4000 B.C. At the same time a second important development, the emergence of bread wheat (Hexaploid wheat, *T. aestivum*) was also taking place. This again arose by hybridisation but this time between an Emmer type wheat, probably cultivated emmer itself and another wild goat grass (*A. squarrosa*) (Fig. 1.1). This hexaploid wheat is widely adaptive and ideal for bread making. It constitutes a major portion of staple diet of humans around the globe. In 1997, wheat was cultivated on 2.26 billion hectares around the world with a production of 6.09 billion metric tonnes (FAO, 1998).

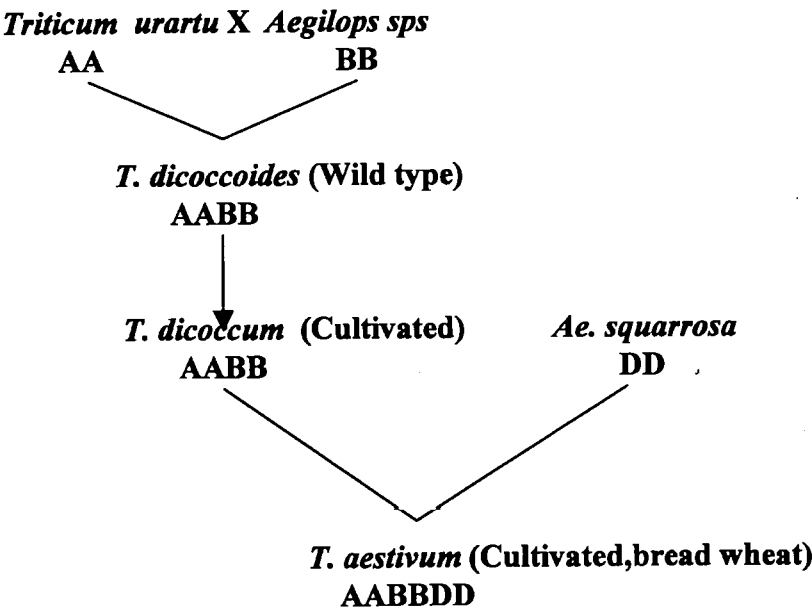


Fig 1.1. The evolution of bread wheat.

1.2 Powdery Mildew

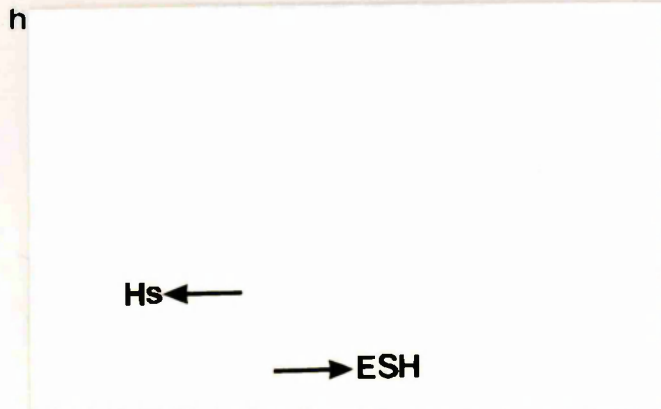
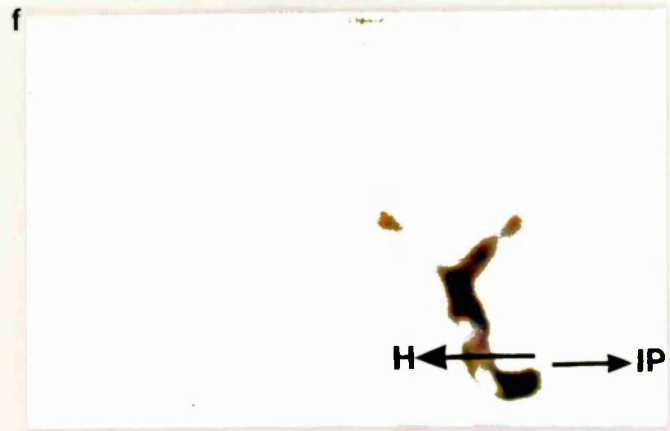
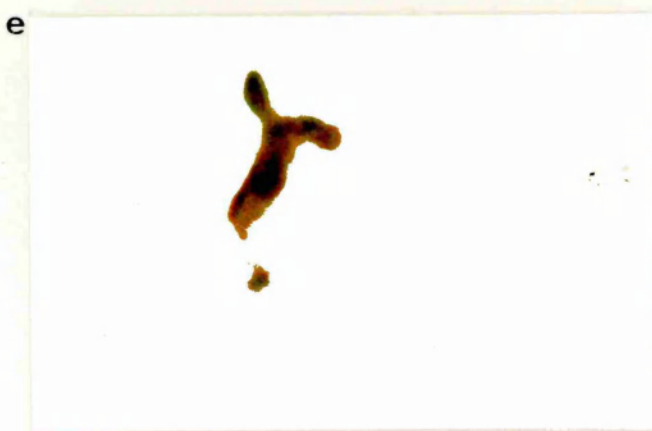
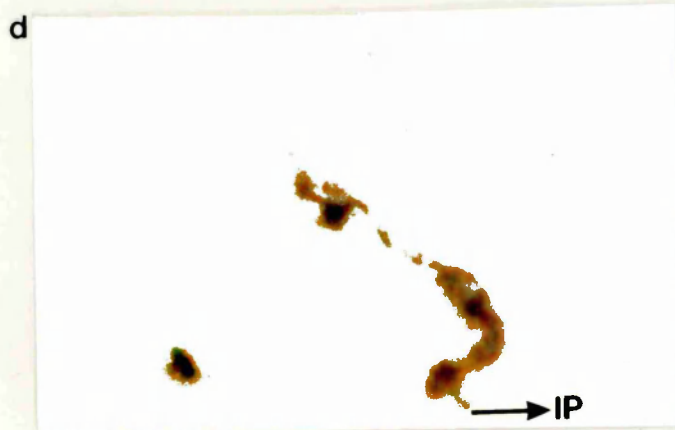
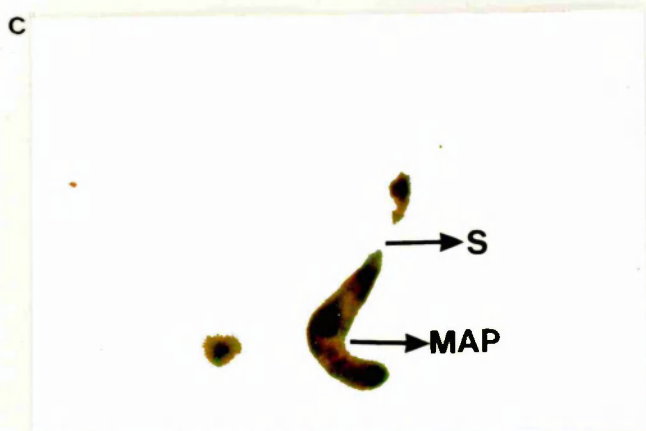
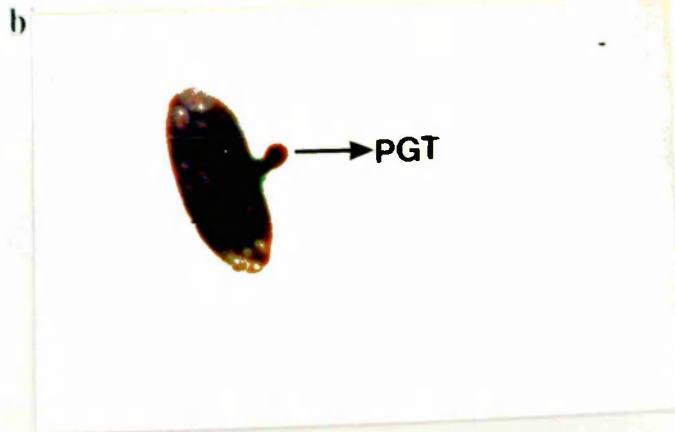
Powdery mildew, caused by *E. graminis* f.sp. *tritici* is an important wheat disease in cool and humid areas of the world causing significant yield losses (Agrios, 1988). In the United Kingdom alone annual yield losses caused by powdery mildew have been estimated at 230 000 tonnes (Cook *et al.*, 1992), equivalent to £22 million loss in revenue from the wheat harvest in 1996-97 (Anonymous, 1997). The fungus is an obligate pathogen, producing superficial mycelium on leaves, stems and ears, significantly reducing the photosynthetic area of the plant. It obtains nutrition from the plant by producing haustoria in the epidermal cells. The mycelium produces short conidiophores on the plant surface. Each conidiophore produces chains of ovoid or round conidia that are carried and dispersed by air currents providing inoculum for further infections. The safest and most economical way of controlling the disease is the incorporation of resistant genes in the breeding material.

1.2.1 Developmental stages of the fungi (Fig. 1.2)

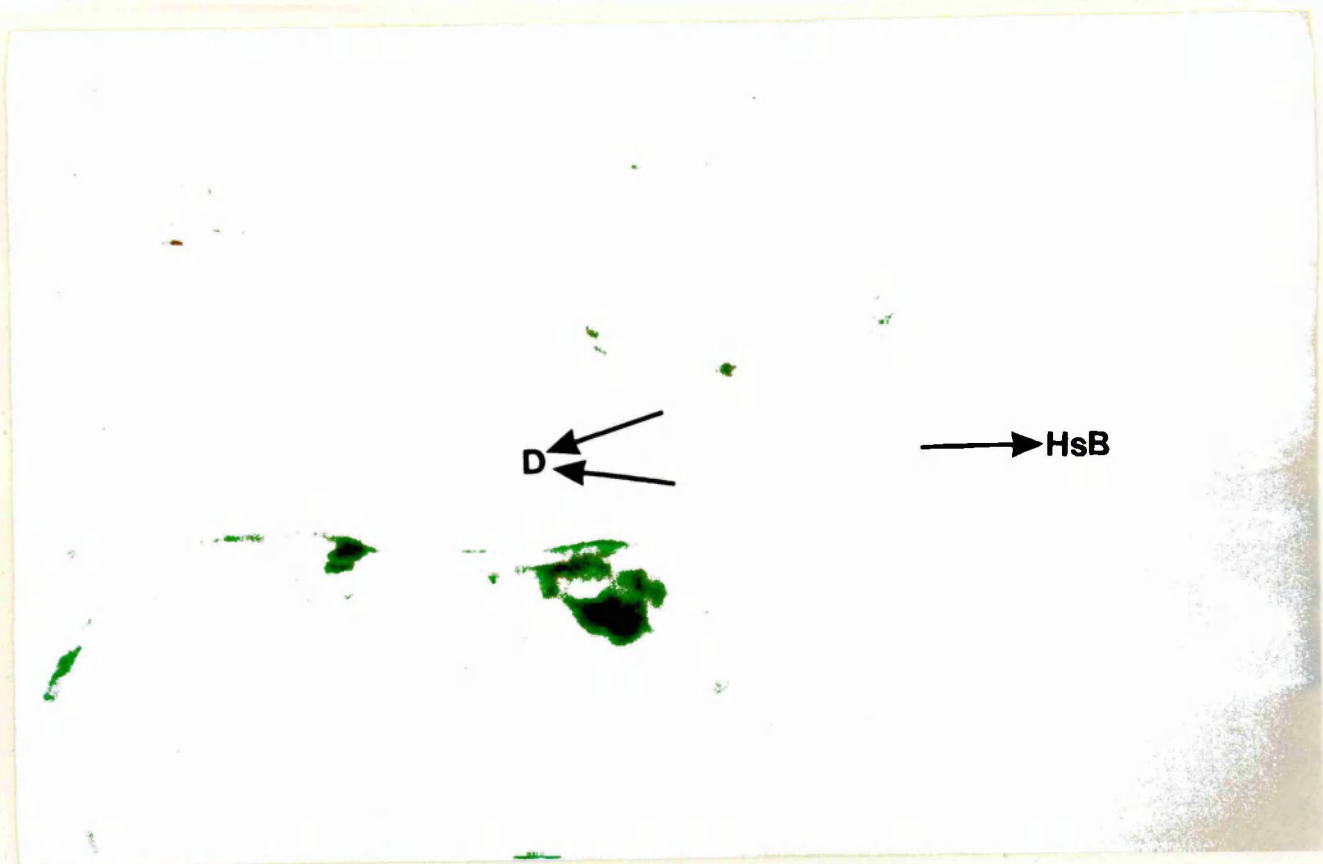
Various developmental stages of *E. graminis* fungi have been well defined and characterised (Boyd *et al.*, 1994a, Ellingboe, 1972, Lyngkjear *et al.*, 1997). The conidium lands on the fresh plant surface and under the right temperature (~20°C) and moisture conditions it germinates, producing a primary germ tube (PGT) within 2-4 hrs (Fig. 1.2b). It has been reported that the PGT plays an important role in host surface recognition and thus triggers the production of a secondary tube called the appressorial germ tube (AGT) (Carver and Ingerson, 1987). The PGT is also thought to provide some anchorage to the spore (conidia) and in gaining access to the host water which may be necessary for AGT formation and elongation (Carver and Bushnell, 1983) subsequently leading to mature appressorium (Fig. 1.2c). An appressorial

Fig. 1.2. Pictographs of powdery mildew (*E. graminis tritici*) development on wheat cultivar 'Hobbit sib' at a magnification of 400 X.

a. ungerminated spore, b. germinated spore with a primary germ tube (PGT), c. mature appresorium (MAP) with a septa (S), d. infection peg (IP) at the end of the appresorial tube, e. secondary lobe, f. IP with a halo (H) on the secondary lobe, g. beginning of a tertiary lobe, h. haustorium (Hs) with an elongated secondary hyphae (ESH), i. Mature haustorium with haustorial body (HsB) and digits (D), j. colony after 72 hours after inoculation.



i



j



infection peg (AIP), produced from a mature appressorium breaches the plant cell wall 10-12 hrs after inoculation. The AIP is the first fungal structure to make contact with the host's cell wall (Fig. 1.2d). At the same time the host responds by the production of papillae (structural barriers around the penetration site (Aist, 1976). Papillae are host cell wall appositions or cytoplasmic aggregates composed of lignins, cellulose and silicon. If the pathogen is unsuccessful in overcoming this physical barrier in the first instance it makes other attempts, producing secondary and tertiary lobes (Fig1.2e-g). Once successful in penetrating the cell wall the pathogen produces a balloon like structure called a haustorium in the epidermal cell of the host (18-24 hrs, after inoculation) (Fig. 1.2h). The haustorium draws nutrition from the host cell by producing several finger like projections called digits (Hazen and Bushnell, 1983, Lyngkjear *et al.*, 1997) (Fig. 1.2i). The mature haustorium enables the fungi to derive nutrients from the host cell, allowing growth of elongated secondary hyphae (ESH). Hyphal growth is supported by the formation of further haustoria, leading to the production of a sporulating colony (4-7 days after inoculation) (Fig. 1.2.j).

1.3. Genetics of Disease Resistance

Biffen (1905) was the first to show Mendelian inheritance for disease resistance working with the yellow rust (*Puccinia striiformis*) fungus on wheat. Flor (1956) working on flax rust proposed the 'Gene for Gene' hypothesis. He proposed that for each gene for resistance (R) in the host there should be a corresponding gene for avirulence (Avr) in the pathogen, where resistance and avirulence are both dominant (Fig.1.3).

	<u>Host</u>	
	<i>R_</i>	<i>rr</i>
	<i>AVR_</i>	
	Resistance	Susceptibility
<u>Pathogen</u>		
	<i>avr</i>	
	Susceptibility	Susceptibility

Fig. 1.3 Representation of Flor's gene for gene hypothesis.

According to the gene for gene relationship, the expression of resistance or susceptibility of the host to a particular pathogen is conditional on the pathogen genotype, and the degree of pathogen virulence observed is conditional on the host genotype. Thus specifically matching gene pairs determine the outcome of any particular host pathogen interaction. Only a resistance gene in the host and matching avirulence gene in the pathogen leads to resistance.

Vanderplank (1963) described resistance (host specific pathogen interactions) as race specific and race non-specific, where race is a particular genotype of the pathogen determined on the basis of its virulence pattern on a set of host genotypes (differentials). Race was first described by Stakman (1916), when he found differences in the virulence of two isolate cultures of *P. graminis* on two wheat genotypes. According to Vanderplank (1963), race specific resistance is generally under monogenic or oligogenic control and he designated it as 'vertical resistance' whilst race non-specific resistance is generally under polygenic control, referred to by him as 'horizontal resistance'. In race specific resistance the host is totally resistant to a particular race of the pathogen, whereas in race non-specific resistance, the host possesses partial resistance to all races of the pathogen. It is now recognised that there

are many exceptions to these generalisations in various host-pathogen interactions e.g. *mlo* governed powdery mildew resistance in barley.

The genome of a plant contains a large number of genes involved in the detection and discrimination of the pathogen and vice versa. So the compatibility of the plant-pathogen interaction is the multitude of series of genes (factors) present both in the host and pathogen with specially matching gene pairs determining the final outcome (resistance levels) (Crute and Pink, 1996). Resistance genes may be under growth or developmental control. Some are active from the seedling stage (Wolters *et al.*, 1993) and remain active throughout the life cycle. Others become active at the adult plant stage described as adult plant resistance (APR) (Heitefuss *et al.*, 1997).

1.4. Resistance gene expression in hexaploid wheat

Hexaploid wheat, composed of the three genomes A, B and D, has a very complex gene expression mechanism. The expression of a particular character, including disease resistance, is dependent on the balance of genes controlling it. It has also been noticed that genes expressed in one of the diploid/ tetraploid progenitors of wheat may not be expressed in the hexaploid situation due to gene suppression. For example seedling resistance of *T. tauschii* to leaf rust was not expressed in the synthetic hexaploid wheats developed by crossing tetraploid wheat with resistant *T. tauschii* accessions (Innes & Kerber, 1994). Also a high phenotypic diversity for resistance to stripe rust in both seedlings and adults of synthetic hexaploid wheats (resistant *T. turgidum* X susceptible *T. tauschii* and resistant *T. tauschii* X susceptible *T. turgidum*) was observed by Ma *et al* (1995). Evaluation of accession lines of *T. boeoticum*, *T. monococcum*, *T. urartu* and durum wheat along with crosses involving these three diploid species and *T. turgidum* (AABB) resulted in the detection of much

variation in the resistance reaction of the amphiploid seedlings (Ma *et al.*, 1997). These results suggest the possibility of some resistance gene suppression.

There are some reports which suggest a possible role for the D genome in the suppression of the resistance genes present on the homcologous chromosomes of the A and B genome. Crosses between *T. dicoccoides*, resistant to leaf and stem rust, and durum (tetraploid wheat, AABB) and bread wheat (hexaploid wheat, AABBDD) resulted in the suppression of resistance in the hybrids with bread wheat whilst the hybrids with durum wheat were resistant (Bai & Knott, 1992). The nullisomic for chromosome 7D of the hexaploid wheat cultivar 'Canhatch' was found to be resistant to several races of stem rust at both the seedling and adult plant stage while the ditelosomic of 7DL was as susceptible as its hexaploid counterpart (Kerber and Green, 1980). It was suggested that the long arm of 7D suppresses the resistance conferred by the genes present on A and B genome and this suppressor was inherited from *Aegilops squarossa* the diploid progenitor of the hexaploid wheat.

All this evidence suggests that the degree of expression of resistance genes at the hexaploid level is dependent on the genetic background in which it occurs as the possible suppressors present on the other genome(s) could inhibit its expression levels. A newly introduced gene, linked with suppressors for other resistance/susceptibility genes may even alter the whole resistance balance of the synthetic genotype resulting in increased susceptibility. Thus breeding for disease resistance in hexaploid wheat requires the expression of resistance genes at higher ploidy level, as the presence of these possible suppressors limit the use of genetic resources. So for a greater success in resistance breeding programmes such suppressor genes could be identified and eliminated and this has already been clearly

demonstrated by the evaluation of aneuploid stocks of various wheat genotypes (Law *et al.*, 1978, Pink *et al.*, 1983, Worland and Law 1991).

Monosomic analysis of 'Hobbit sib' by Worland and Law (1991) showed that 12 monosomic lines had significant deviations from the euploid control for yellow rust resistance. Of these 12 chromosomes, seven carried genes for improving the resistance ranked in the order 5BS-7BS > 6B > 2D > 4A > 2B > 1A > 2A while 5 promoted susceptibility and ranked as 5D > 5BL-7BL > 4B > 4D > 3B.

Altering the chromosome dosages, especially arms of group 5, had a significant effect on the overall resistant state of adult 'Chinese Spring' and 'Hobbit sib' to yellow rust and powdery mildew. Evaluation of ditelosomics for chromosome 5 showed the short arms had genes which increased resistance while the long arms had the opposing genes (Pink *et al.*, 1983, Worland and Law, 1991).

1.5 Elimination of suppressor / susceptibility genes

Elimination of suppressor/susceptibility genes should lead to enhanced resistance in the host. Various methods have been suggested:

1.5.1. Chromosomal substitution

Chromosomes carrying suppressor/ susceptibility genes could be substituted with their counterparts from other genotypes. Pink *et al.*, 1983 observed a significant variation in the yellow rust and powdery mildew resistance levels of 'Chinese spring' intervarietal substitution lines for group five chromosomes and concluded this variation to be allelic. A similar study for yellow rust resistance was done by Worland and Law 1991, with 'Hobbit sib'-'Bezostaya 1' substitution lines. Analysis of substitution lines indicated 6 'Bezostaya' chromosomes, ranked as 5BS-7BS > 5A > 4A > 2D > 3A > 3D, carried genes for lowering the disease resistance in 'Hobbit sib'

(substitution) either because they were carrying weaker alleles for resistance or stronger alleles for the promotion of susceptibility, whilst seven 'Bezostaya 1' chromosomes were more efficient in promoting resistance than their 'Hobbit sib' homologues; ranked as 5BL-7BL > 6B > 5D > 7D > 2 A > 4B > 4D in order of increased efficiency at promoting rust resistance. Thus it was concluded that 'Bezostaya 1' lacked the four major potent genes for the promotion of susceptibility as carried by 'Hobbit sib' on chromosomes 4B, 4D, 5BL-7BL and 5D.

1.5.2 Selection of non suppressor alleles

LrT2, a leaf rust resistance gene, was found to be associated with stem rust resistance in the backcross lines of 'Thatcher' bread wheat cultivar (Dyck, 1987). Genetic and cytogenetic studies indicated that this gene was present on 7D. This 7D chromosome was documented to carry suppressor genes for stem rust resistance (Kerber & Green, 1980) in the cultivar 'Canthatch'. Substitution of the 7D chromosome carrying LrT2 gene in the 7D nullisomic of 'Canthatch' showed no loss of resistance. The lack of susceptible progeny to stem rust at F2 indicated that LrT2 was a non suppressor allele at the 'Canthatch' 7D suppressor locus. Selection for non suppressor alleles was also suggested by Ma *et al.*, 1995 when a high variation in resistance to stripe rust was observed in 74 synthetic hexaploid wheat lines (resistant *T. turgidum* X susceptible *T. tauschii* and susceptible *T. turgidum* X resistant *T. tauschii*) as seedlings and adult plants.

1.5.3 Mutagenesis

Use of artificial mutation has been proposed by several workers to eliminate suppressors / susceptibility genes from the wheat genome (Kerber, 1991, Kerber and Aung, 1995, Ma *et al.*, 1995, Law *et al.*, 1978, and Pink *et al.*, 1983). Mutagen

treatment of the susceptible genotype has been helpful in the production of several resistant lines. Williams *et al.*, (1992) treated 'Canhatch' with ethyl methanesulfonate and identified 15 stem rust resistant mutants. Similarity among these mutants in their resistance response to 13 races of stem rust indicated that each of these mutants had a similar genetic alteration resulting in an inactivation or deletion of the suppressor gene. Crosses between the mutant and 'Chinese spring' monosomic 7D or 'Chinese spring' 7DS ditelosomic indicated 'Canhatch' might have three recessive genes for resistance which were inhibited by the suppressor gene. They suggested these mutant lines might permit the use of these suppressed genes for stem rust resistance to breed resistant cultivars.

In 1982, 11000 seeds of 'Hobbit sib' were treated with fast neutrons and after three generations of selection, 20 lines showing improved levels of resistance and trueness to parent type were retained (Worland and Law, 1991). These 20 lines were further characterised and various deletions in them were identified by monosomic analysis and molecular markers RFLP (Restriction Fragment Length Polymorphism) (Worland and Law, 1991). Most of the deletions were identified on the chromosomes thought to carry genes for suppressing resistance. Although lines were selected for their resistance to yellow rust, some also had improved resistance to brown rust and powdery mildew (I3-48 and I3-54). Carrying this work forward Howie (1997), using a combination of RDA (Representational Difference Analysis) and RFLP markers, detected deletions in twelve of the 'Hobbit sib' mutants some, of which were not known previously. He identified a linkage of deletion on 4DL with resistance in the segregating population of I3-48 X 'Rialto'. Segregating plant material from other mutant X Rialto crosses was also found to be resistant but there was no linkage or

association with the known deletions in the parent mutant genotype. Thus the presence of unknown deletions in the mutant genotypes was suggested.

1.6. Resistance to *E. graminis*

Histological studies of *E. graminis* (pathogen) and cereal (host) interaction have been widely reported. This has helped in identification and characterisation of resistance genes specifically restricting the fungal growth at different stages. Race non-specific *mlo* alleles provide papilla mediated quantitative resistance in barley (Aist *et al.*, 1988, Wolters *et al.*, 1993). In a few cases papilla production is accompanied by the accumulation of various phenolic compounds restricting the growth and penetration at the appressorial germ tube stage leading to a hypersensitive cell death (HR) (*Mlg*, Gorg *et al.*, 1993). If the fungus succeeds in overcoming the papillae mediated resistance barrier it leads to the production of haustoria inside the host epidermal cell. At the same time various antifungal compounds or metabolites accumulate in the host cell. Excessive production of these substances could lead to host cell death (HR) leading to immediate arrest of the pathogen during haustorial formation (*Mla*₁, *Mla*₁₂ in barley) (Gorg *et al.*, 1993, Koga *et al.*, 1990). Genetic resistance to this pathogen can either be race specific (*Mla*₁, *Mla*₁₂, *Mlg*) or race non-specific (*mlo* alleles).

1.7. Host – Pathogen interaction: Resistance *per se*

Plants come in frequent contact with potential pathogens yet disease results from relatively few of these exposures. Plants may be able to recognise and resist pathogens either because:

- The plant is unable to support the niche requirements of a potential pathogen and is thus a non-host.

- The plant possesses pre-formed structural barriers or toxic compounds that confine successful infections to specialised pathogen species only.
- Upon recognition of the attacking pathogen, defence mechanisms are elaborated and the invasion remains localised.

All the three interactions are said to be incompatible but only the latter resistance mechanism depends on induced responses in line with the 'gene for gene' hypothesis resulting in incompatibility (HR) and hence leading to immediate arrest of the pathogen (Gorlach *et al.*, 1996, Greenberg *et al.*, 1994, Lamb *et al.*, 1989, Ross, 1961, Ryal *et al.*, 1996). Successful pathogen invasion and disease ensue if the preformed plant defences are inappropriate, the plant does not detect the pathogen or the activated defence responses are ineffective or slow (Gregersen *et al.*, 1993, Kossack and Jones, 1996, Osbourn, 1996, Scott, 1993). Following the 'gene for gene' concept a series of resistance genes in plants and avirulence genes in the pathogen have been identified and cloned (Baker *et al.*, 1997).

Another type of disease resistance mechanism which can either be associated with the hypersensitive reaction (HR) or totally independent (non-host pathogen interaction) is acquired resistance (Bowles, 1990, Draper, 1997, Greenberg *et al.*, 1994, Ross, 1961). Acquired resistance results from a HR or a non-host pathogen interaction or infection with a necrotizing pathogen and can lead to resistance to the inducing agent (pathogen) as well as to a broad spectrum of viral, fungal and bacterial pathogens. This resistance response can either be local or systemic. Locally induced resistance is limited only to the specific area of pathogen attack whilst systemic resistance takes place throughout the plant system (Systemic acquired resistance, SAR).

Both HR and SAR depend on interaction between the resistance gene product in the plant and corresponding avirulence gene in the pathogen. Plant resistance genes are postulated to encode receptors that interact directly or indirectly with elicitors (ligands) produced by pathogen (Baker *et al.*, 1997) upon pathogen recognition. This signal transduction has been reported to trigger defence gene activation; hydrogen peroxide (H₂O₂), phenylalanine ammonia lyase (PAL) and salicylic acid (SA) are thought to be the important components (Becker *et al.*, 1998, Chen *et al.*, 1995, Draper, 1997 Greenberg *et al.*, 1994, Peltonnen *et al.*, 1998, Ryals *et al.*, 1994, Ryals *et al.*, 1996, Wu *et al.*, 1997).

Associated with the resistance response (SAR or HR) in plant species is a set of genes encoding pathogenesis related proteins (PR proteins) or SAR genes. These are activated in a co-ordinated manner and most likely play an important role in the onset and maintenance of SAR (Baker *et al.*, 1997, Gregersen *et al.*, 1993, Keen, 1992, Ryals *et al.*, 1994, Scott *et al.*, 1990, Ward 1991). These PR proteins were first studied by Van Loon and Vankammen (1970) in tobacco reacting hypersensitively to tobacco mosaic virus. Since then a series of PR proteins has been identified and reported in several crop species. There are two types of PR proteins: acidic and basic forms.

Acidic PR proteins are selectively extracted with buffer at pH 2.8 and are resistant to endogenous proteases. These generally accumulate in intercellular spaces and are usually associated with resistance to pathogen infection, SA being the mediator. Basic PR proteins are generally induced during wounding or injury response with jasmonic acid as the mediator, and are not found in the intercellular

spaces but are present in vacuoles and are constitutively expressed in roots (Linthorst, 1991 and Ohahsi and Ohshima, 1992).

1.8. PR proteins and resistance

Many of the genes coding for PR proteins have been cloned and characterised according to their specific functions. Several of these PR proteins have anti-pathogenic activities or show an enhanced accumulation during resistance reaction. Studies conducted on isogenic lines differing for the resistant gene loci in barley showed a rapid increase in the accumulation of various PR proteins in the resistant lines in comparison to the susceptible ones (Boyd *et al.*, 1992, Boyd *et al.*, 1995, Bryngelsson and Collinge 1991, Davidson *et al.*, 1987 and Davidson *et al.*, 1988,). A comparison of mRNA induction by Davidson *et al* (1988) between the resistant and the susceptible near isogenic lines showed a 3 fold higher accumulation of two mRNA's in all the resistant cultivars 24-48 hrs after inoculation. They concluded that the genes for resistance function as regulators for the synthesis of common host mRNA's during the early stages of infection.

A cumulative induction in the transcripts of various PR proteins, regardless of the presence or absence of a resistance gene, has also been observed. Brynelgesson and Collinge (1991) studying the hypersensitive response of barley to *E. graminis* found enhanced levels of transcripts of PR1 in both compatible and incompatible reactions after inoculation. A similar pattern of induction in the enzyme activities of peroxidase and β -1,3, glucanase was observed in the resistant and the susceptible lines of maize after inoculation with *Exserohilum turcicum* by Shimoni *et al.*, (1996). However, studies done by Boyd *et al* (1994a), Boyd *et al* (1994b) and Bushnell and Liu (1994) on incompatible disease reaction (HR response, avirulent pathogen)

indicated an early synthesis and higher transcript levels of various PR proteins in comparison to a virulent pathogen.

PR proteins can also be induced in non-host pathogen interactions (Bull *et al.*, 1992, Dudler *et al.*, 1991, Gregersen *et al.*, 1990, Gregersen *et al.*, 1994, Schweizer *et al.*, 1989) and by chemical and other stresses (UV light, BTH, Cytokinins) (Gorlach *et al.*, 1996, Pinto and Ricardo, 1995, Sachaffarth *et al.*, 1997, Siefer *et al.*, 1996) leading to an induced resistant state in the host to subsequent infection even against compatible (host specific) pathogens. Table 1.1 lists various PR proteins identified and cloned from cereals.

Table. 1.1 Various PR proteins identified in cereals in response to pathogen invasion along with their biochemical properties.

Families	Biochemical Properties	Crop species	References
PR-1	Unknown	Barley	Muradov <i>et al.</i> , 1993 Bryngelsson <i>et al.</i> , 1994 Stevens <i>et al.</i> , 1996
PR-2	β ,1-3,glucanase	Barley	Malehorn <i>et al.</i> , 1993
PR-3	Chitinase	Wheat	Lia <i>et al.</i> , 1994 Garthorf <i>et al.</i> , 1997
PR-4	Win proteins	Wheat	Caruso <i>et al.</i> , 1996
PR-5	Thaumatococin like	Wheat	Rebmann <i>et al.</i> , 1991(a)
		Wheat	Reimann <i>et al.</i> , 1993
PR-6	Proteinase inhibitor	Barley	Brandt <i>et al.</i> , 1992
PR-9	Peroxidase	Wheat	Rebmann <i>et al.</i> , 1991(b)
		Rice	Reimann <i>et al.</i> , 1992
Novel types	Glutathione-S-transferase	Wheat	Dudler <i>et al.</i> , 1991
	pWIRI	Wheat	Bull <i>et al.</i> , 1992
	Thionin	Wheat	Castagnaro <i>et al.</i> , 1995
	Oxalate Oxidase	Barley	Dumas <i>et al.</i> , 1995 Wei, <i>et al.</i> , 1998
	Lipid transfer	Barley	Molina <i>et al.</i> , 1993

1.9. Mutant mimics

There are some reports of mutants mimicing the resistant reaction (SAR gene activation) in totally sterile (disease free) conditions. Wolters *et al* (1993) identified three isogenic lines of barley for the *Mlo* locus; *mlo1*, *mlo2* and *mlo5* which undergo spontaneous formation of cell wall appositions under disease free conditions. On further analysis a high level of chemical and structural similarity was observed

between these spontaneously formed appositions with the pathogen triggered ones. Similarly six *Arabidopsis* mutants forming spontaneous necrotic lesions under disease free conditions were identified by Dietrich *et al* (1994). The necrotic lesions in five of these mutants correlate with the expression of various histochemical and molecular markers of plant disease responses and with the expression of a PR1 gene. On testing these mutants against fungal infection they found that four mutants exhibited reduced or altered hyphal growth and reduced oospore production. A similar constitutive expression of SAR genes in mutagenised *Arabidopsis* (transformed with a reporter gene comprising the promoter of β -1,3 Glucanase- encoding PR gene (BGL-2) and the coding region of β -glucuronidase) was observed by Bowling *et al* (1994). They identified one transgenic mutant, cpr-1, with elevated expression of endogenous PR genes, BGL2, PR1 and PR5. Further analysis of this mutant showed that it was resistant to both the fungal pathogen *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* pv *maculicola*. In addition, it had higher levels of SA in comparison to the wild type. So by artificial mutagenesis we can also induce the resistance gene responses in the plant species even under totally disease free conditions. This resistance gene response could be under some developmental regulation as there is no host pathogen interaction involved (Wolters *et al.*, 1993).

1.10 Project Objectives

The aim of this project was to study resistance to powdery mildew in mutants of 'Hobbit sib' known to contain deletions.

Worland and Law (1991) and Howie (1997) evaluated resistance only on the flag leaves at the adult plant stage of the 'Hobbit sib' deletion series (I3-21, I3-27, I3-32, I3-43, I3-48, I3-54 and I3-58). Moreover, the resistance levels for some mutants

were inconsistent (Table. 2.8). So the first objective of the present study was to investigate powdery mildew resistance at different growth stages on individual leaves. This would help in characterising the type and timing (APR or seedling resistance or both) of resistance gene activation in the resistant mutants.

Secondly, the development of the powdery mildew fungus in the resistant mutants would be examined microscopically to determine differences in development from infection of the wild type, 'Hobbit sib'.

Thirdly constitutive expression of various PR proteins in the resistant mutant(s) would be determined by measuring their mRNA expression levels in disease free plants and comparing this with those in 'Hobbit sib'.

Hobbit sib, a close relative of Hobbit (high yielding winter feed wheat, bred by PBI) originally had effective APR and seedling yellow rust resistance (Yr1, Yr2, Yr3a/4a and Yr14). However this resistance was overcome in 1975, with the evolution of new yellow rust race 41E136 (Taylor *et al.*, 1981, Worland and Law unpublished). But 'Hobbit sib' did not become fully susceptible because of some background resistance. In 1975-76 it was tested for mildew resistance in the 'Annual Physiological Race Survey' but no race specific resistance genes were assigned to it. After its mutagenesis in 1982 a series of deletion mutants possessing high levels of APR to yellow and brown rusts were selected. However only limited and sometimes contradictory data were obtained on APR to mildew (Howie, 1997).

Disease resistance in plants is controlled by a combination of various genes. Some of these may be under some growth or developmental regulation expressing only at the seedling stage (seedling resistance) or adult plant stage {adult plant resistance, (APR)}. Seedling resistance generally remains active throughout the plant life cycle though its effectiveness at the adult plant stage might decrease. So a better understanding of the developmental control and maintenance of resistance at various growth stages is required.

In line with all this, seven mutants (I3-21, I3-27, I3-32, I3-43, I3-48, I3-54 and I3-58) and wild type 'Hobbit sib' were tested for their resistance to *Erysiphe graminis tritici* at different adult and seedling stages on individual leaves.

2.1 Material and Methods

2.1.1 Adult plant resistance (APR) test

Seven mutants (I3-21, I3-27, I3-32, I3-43, I3-48, I3-54 and I3-58) and wild type 'Hobbit sib' laid in a randomised block design with six replications were tested for their APR in autumn of 1997.

2.1.1.1. Raising of plant material

The seedlings of all the eight genotypes were raised in 1 % MS sucrose medium in test tubes after surface sterilisation of the seeds with 10 % sodium hypochlorite solution. All the seed material was first incubated at 5°C in the dark for 2 days to break the seed dormancy before moving them to 16 hrs day length and 18°C day temperature for two weeks. After this the plant material was vernalised for eight weeks at 5°C and 8 hrs day length and later potted on, in John Innes No.2 compost with one seedling per 7.5 cm diameter pot. These pots were moved to a clean glasshouse fitted with fans and maintained under positive air pressure. The plant material was kept in the clean glasshouse for eight weeks after vernalisation plants were at the earing stage (growth stage 51-59, according to Zadoks *et al.*, 1974). These plants were then shifted to another glasshouse using a clean mobile cubicle cabinet to prevent any contamination during transaction. The test plants were inoculated in the new glasshouse with freshly produced, mildew spores from 'Cercospora' seedlings.

2.1.1.2 Source and virulence spectra of inoculum:

Five to seven 'Cercospora' seedlings (highly susceptible wheat genotype having no known resistance genes) in five 7.5 cm diameter pots were sown five weeks before the test inoculation date. These 'Cercospora' seedlings were kept in a separate compartment in the glasshouse used for disease tests. At rabbit ear leaf stage (growth stage 11, according

to Zadoks *et al.*, 1974) these seedling pots were moved out and left at different locations in the wheat field for 24 hrs, allowing them to become infected with a sample of the prevalent races in the field. The pots were moved back to the compartment and after a week, sporulating mildew colonies were observed on the seedlings. The mildew colonies on the seedlings multiplied and provided inoculum for about two weeks. In the mean time another batch of 'Cерco' seedlings was raised and was inoculated from the inoculum produced by the mildew colonies on the previous batch of 'Cерco' seedlings. The cycle was repeated and in this way mildew inoculum was multiplied by infecting successive batches of 'Cерco' seedlings. Inoculations were done by shaking some of these heavily infected 'Cерco' seedlings over the next batch. The virulence genes present in the mildew population were determined by differential testing alongside the test material. The mildew population had virulence genes for 2, 3c, 4b, 5, 6, 8, 11 and *Mli* loci.

2.1.1.3 Inoculation

Ten pots of 'Cерco' seedlings with heavily sporulating colonies were used for inoculating the test material for the APR test. For the first two days after inoculation, high relative humidity was maintained by flooding the bench surface with water to enhance the spore germination.

2.1.1.4 Disease assessment:

Weekly mildew scores were assessed on the flag leaves (mean of 5 plants per treatment) by estimating the percentage of the total area infected. First mildew scores were made two weeks after inoculation and continued for 3 weeks. Average scores over 4 weeks for each genotype were computed and analysed by using SAS statistical package.

2.1.2 (a) Expression of resistance at different growth stages-I

2.1.2. (a) 1. Plant material:

To study the expression of resistance at different growth stages (GS), two resistant mutants; I3-48 and I3-54 and the wild type 'Hobbit sib' were tested at five growth stages (GS); GS 13.21 (pre-vernalised seedlings), GS 13.21 (post-vernalised seedlings) (Zadoks *et al.*, 1974) and at six weeks, seven weeks and eight weeks after vernalisation. Sowing date was varied so that plants at different growth stages would be available together on the date when inoculation was planned. Previous notes on rate of development in the greenhouse were used as a guide for the sequential sowing.

The plants were raised as in section 2.1.1.1 except for pre-vernalised seedlings treatment. For these seven seeds were sown in each 7.5 cm pot and were first incubated at 5°C in the dark for 2 days and then moved directly to the clean glasshouse. Five seedlings were retained when they were two weeks old. The plant material for the four post-vernalised growth stages was transplanted in John Innes No.2 compost. For the post-vernalised seedlings five plants were transplanted into a 7.5 cm pot one week before the test inoculation date to allow acclimatisation to the changed growth conditions. Plant material for testing at the adult plant stages was transplanted, with one seedling per 7.5 cm pot and was grown for six weeks, seven weeks and eight weeks after vernalisation. Variation in the rate of growth of the three adult plant stages was observed so it was decided to group them according to their time intervals after vernalisation rather than Zadoks growth scale.

This test was carried out in autumn 1997 two weeks after the APR test. The plant material was laid out in a split plot design having three replications with genotypes on main plots and growth stages as sub plots.

2.1.2. (a) 2 Inoculation and disease assessment:

Inoculation and disease assessment procedures were the same as in sections 2.1.1.3 and 2.1.1.4. The same mildew population maintained on the 'Cerco' seedlings was used. However only the flag leaf was scored on the adult plants whereas for seedlings, infection was assessed on a whole plant basis. Average scores over four weeks for each treatment were computed and used for statistical analysis according to the 'Genstat' computer package.

2.1.2 (b) Expression of resistance at different growth stages-II

2.1.2 (b) 1 Plant material:

To check the consistency of the results from the previous test, I3-48, I3-54 and 'Hobbit sib', were tested at four different growth stages; pre-vernalised seedlings (GS 13.21), post vernalised seedlings (GS 13.21), booting (GS 40-49) and earing (GS 50-59). The plants used for the adult plant stages were selected from three batches of adult plant material (raised as in section 2.1.2.(a)1). Five plants per treatment were arranged in a completely randomised factorial design which gave a more precise estimation of variation between the different variance components than the split plot design.

2.1.2. (b) 2. Inoculation

This test was conducted in the early summer of 1998. The mildew inoculum was multiplied on 'Cerco' seedlings as in section 2.1.1.2. The mildew population this

time had the virulence for genes 1, 2, 3c, 3d, 4b, 5, 6, 8, 9, 11, *Mld* and *Mli* loci. The inoculation procedures were the same as for the two previous experiments.

2.1.2 (b) 3. Inoculum density

Average spore density and percentage spore viability for this experiment was also determined. Five petri plates, each containing about 20 ml of tap water agar, were placed in each replicate at the same level as the test material during inoculation. After 10-15 minutes (for the spore settling time) the plates were covered with lids and kept overnight along with the test material. The following morning germination percentage was estimated under a light microscope at 128X magnification. Counts were made in 10 fields of view for each plate.

2.1.2. (b) 4. Disease assessment

Disease was assessed using three individual leaves per treatment; the youngest (flag leaf for the adults while third in the seedlings), second (middle) and oldest (third from the top in adults and first in seedlings) were taken. Disease assessments were made for one disease cycle by scoring each individual leaf at 10, 12 and 15 days after inoculation. Analysis of variance (using SAS) for each scoring period was first done separately. As the trend (resistance levels) was found to be similar amongst all the scoring intervals and variances were approximately equal, average scores for each leaf at individual growth stage were computed and used for the final analysis.

2.1.3. (a) Seedling resistance test I.

2.1.3. (a)1 Plant material

To investigate the expression of resistance at different stages in seedling development, all the seven mutants and the wild type were tested at three different seedling stages; second leaf (third leaf equal length of second leaf, GS 12), third leaf (fourth leaf equal

length of third leaf, GS 13.21) and fourth leaf (fifth leaf equal length of fourth leaf, GS 14.22) (Zadoks *et al.*, 1974).

The plant material was raised as pre-vernalised seedlings in section 2.1.2(a).1 and laid out in a completely randomised factorial design with three replications. This test was carried out in summer 1998 and the same mildew population used for expression of resistance at different growth stages-II was multiplied and used. The average spore density and percentage spore viability for the experiment were also measured. Scoring and data analysis was done in the same way as in section 2.1.2 (b). 3, except individual leaves were assessed.

2.1.3 (b). Seedling resistance test II

To check the consistency of the results from the seedling resistance test I, and with slight modifications, seedling resistance test II was conducted. In seedling resistance test I parts of one of the replication had a higher inoculum dosage and there was variation in spore viability. So this time all the replications were inoculated by having the test plant material closer together and using same stage of infected spreader (Cercospora) seedlings for each replication. All the eight genotypes at three growth stages; first (GS 11), second (GS 12) and third leaf (GS 13.21) (Zadoks *et al.*, 1974), laid out in a completely randomised factorial design with three replications, were tested using the same mildew population as for the previous seedling test. The virulence genes in the mildew population were confirmed by repeating differential testing. Similar experimental procedures as in seedling resistance test I for calculating average spore density and percentage spore viability and data analysis were followed.

2.2 Results

2.2.1 Adult Plant Resistance Test

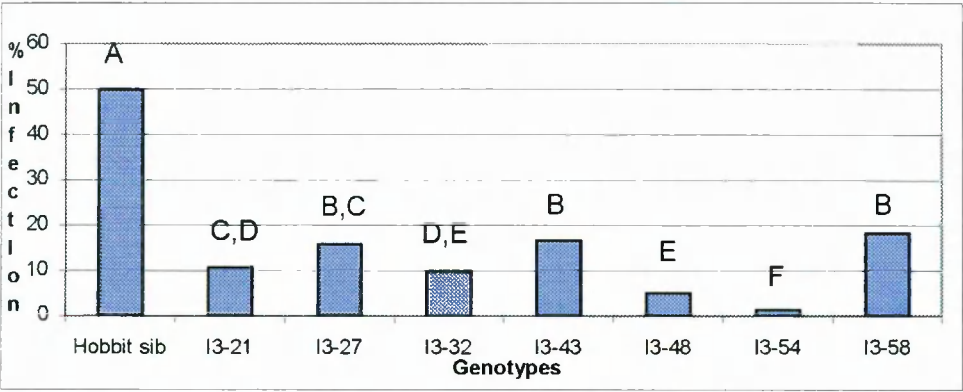
Seven mutants and 'Hobbit sib' were tested for their resistance to *E. graminis tritici* at the adult plant stage. The results from the analysis of variance (Table.2.1) showed a highly significant F value (Probability, $P = 0.0001$) for genotypes while for the replications the F value was non-significant. This indicates that variation amongst the genotypes for their APR levels is largely genetic.

Table. 2.1 Analysis of variance for APR.

Source	Degrees of freedom	Sum of Square	F Value	P
Genotype	7	1330.46	65.15	0.0001
Replication	5	17.98	0.88	0.5043
Error	35	20.42		

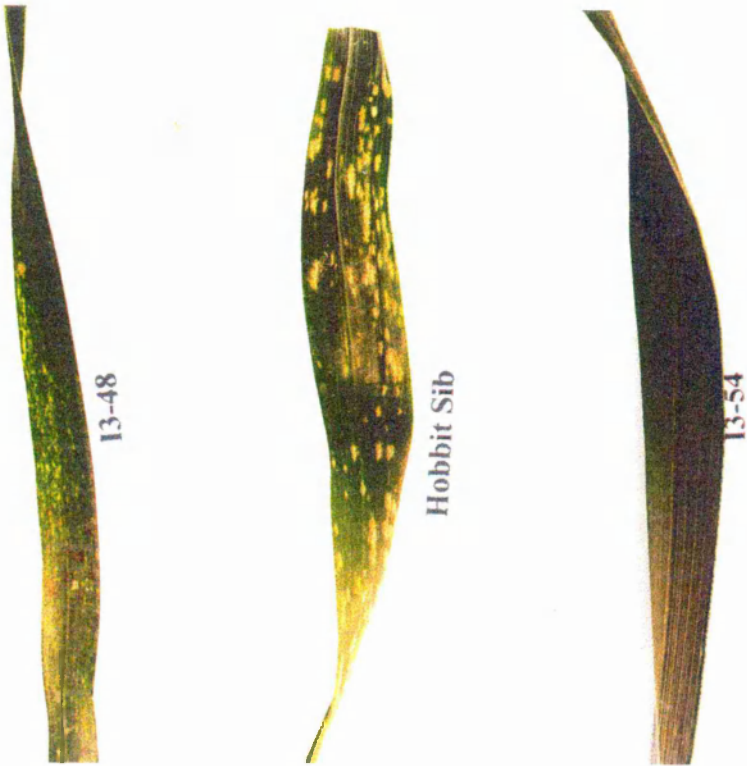
From the mean mildew scores (Least significant difference = 5.3, t value = 2.03) of the genotypes over all the replications, the genotypes could be grouped into six classes (Fig. 2.1a). The wild type with a mean score of 50 was the most infected genotype while I3-54 had the lowest infection levels with a mean score of 1.5 (Fig. 2.1b). The other 6 mutants had intermediate resistance levels and were grouped into 4 overlapping classes, according to 'Duncan's multiple range test' with I3-58 having a maximum score of 18.33 while I3-48 had the minimum of 5.16 (Class I: I3-58, I3-43, I3-27; Class II: I3-27, I3-21; Class III: I3-21, I3-32 and Class IV: I3-32, I3-48). The APR levels from this experiment indicated wild type 'Hobbit sib' and mutant I3-54 to be the most susceptible and resistant genotypes respectively.

Fig. 2.1a Mean percentage mildew infection on flag leaves of Hobbit sib mutant series, Adult Plant Resistance test.



Mean scores with same letter are not significantly different at P = 0.05.

Fig. 2.1b. Powdery mildew infection on flag leaf of three wheat genotypes.



2.2.2 (a). Expression of resistance at different growth stages-I

Hobbit sib (susceptible) and the two genotypes showing highest resistance in APR test, section 2.2.1 (I3-54 and I3-48) were inoculated at five different time periods in relation to vernalisation to study the effect of maturity on resistance. All the previous resistance studies for this mutation series have been carried out only at adult

plant stage so it was decided to investigate the resistance levels at the seedling stage also. Analysis of variance indicated highly significant ($P= 0.01$) F values for genotype, growth stage and genotype X growth stage (Table. 2.2). Hobbit sib had the maximum mean score of 52.67 over all the growth stages. Amongst the growth stages, the post-vernalised seedlings had the higher infection score (24.11) over all genotypes. The effect of growth stage on resistance expression among genotypes can be seen in Fig. 2.2 (a). Hobbit sib had significantly ($P= 0.05$) higher infection levels than the two mutants at all the five growth stages. The two mutants were significantly different from each other only at pre vernalised seedling stage ($P= 0.05$). The resistance in I3-48 and I3-54 was effective at all the growth stages of plant development tested.

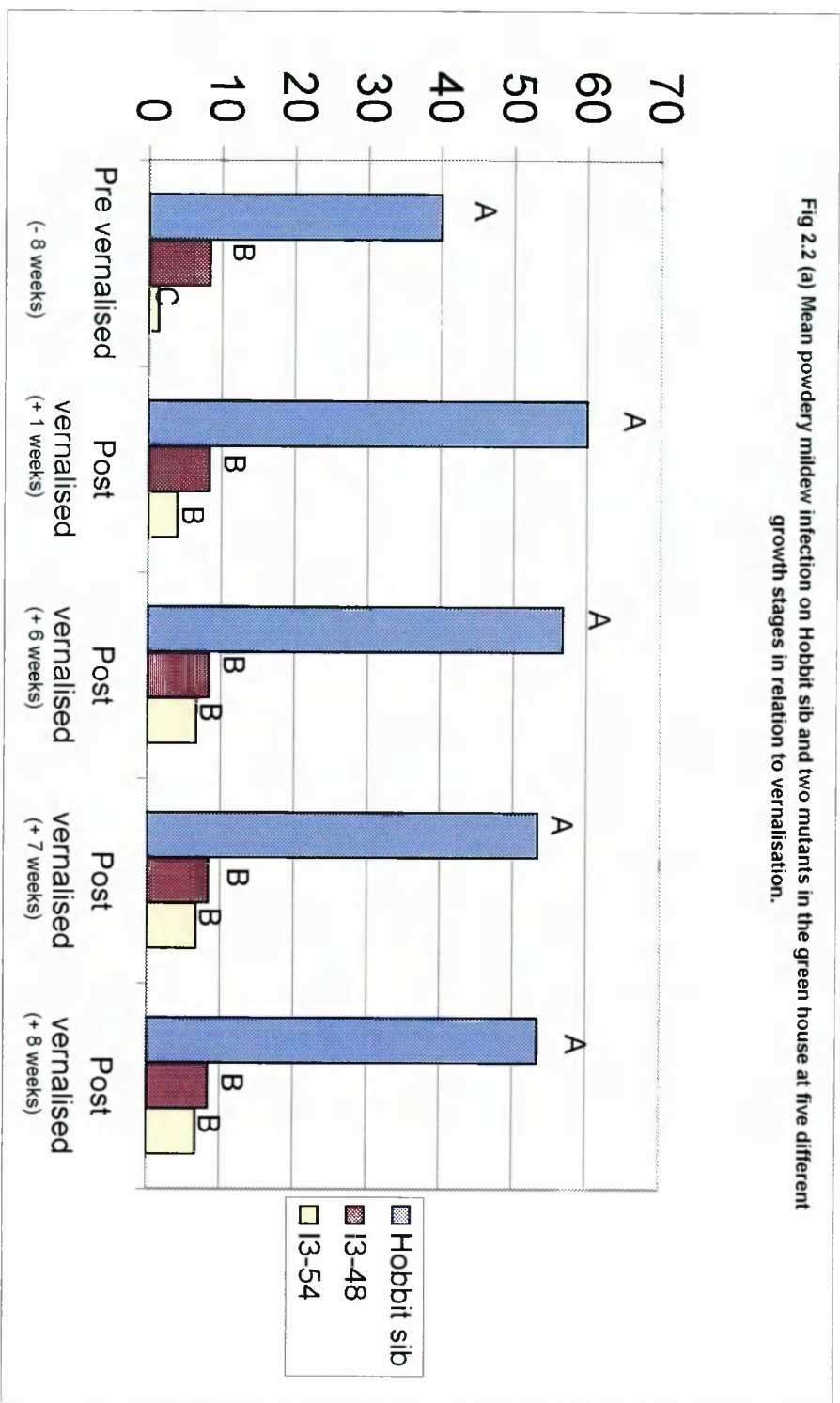
Table.2.2 Analysis of variance table for testing developmental control of resistance.

Source	Degrees of freedom	Mean Square	F Value	P
Replication	2	43.89	0.56	
Genotype (A)	2	10604.69	135.86	0.001
Error (a)	4	78.06	5.48	
Growth stage (B)	4	87.47	6.14	0.002
A X B	8	51.47	6.61	0.007
Residual	24	14.25		
Total	44			

2.2.2 (b) Expression of resistance at different growth stages – II

This experiment was a repeat of the previous one with some modifications. This time percentage infection on three individual leaves per treatment was assessed after only one disease cycle (fifteen days after inoculation). At the same time, average spore density (4.7/mm²) and percentage spore viability (48.3 %) were also calculated. Analysis of variance for each individual leaf was done separately. The F values for

Fig 2.2 (a) Mean powdery mildew infection on Hobbit sib and two mutants in the green house at five different growth stages in relation to vernalisation.



X axis: Growth stages **Y axis: Mean infection**

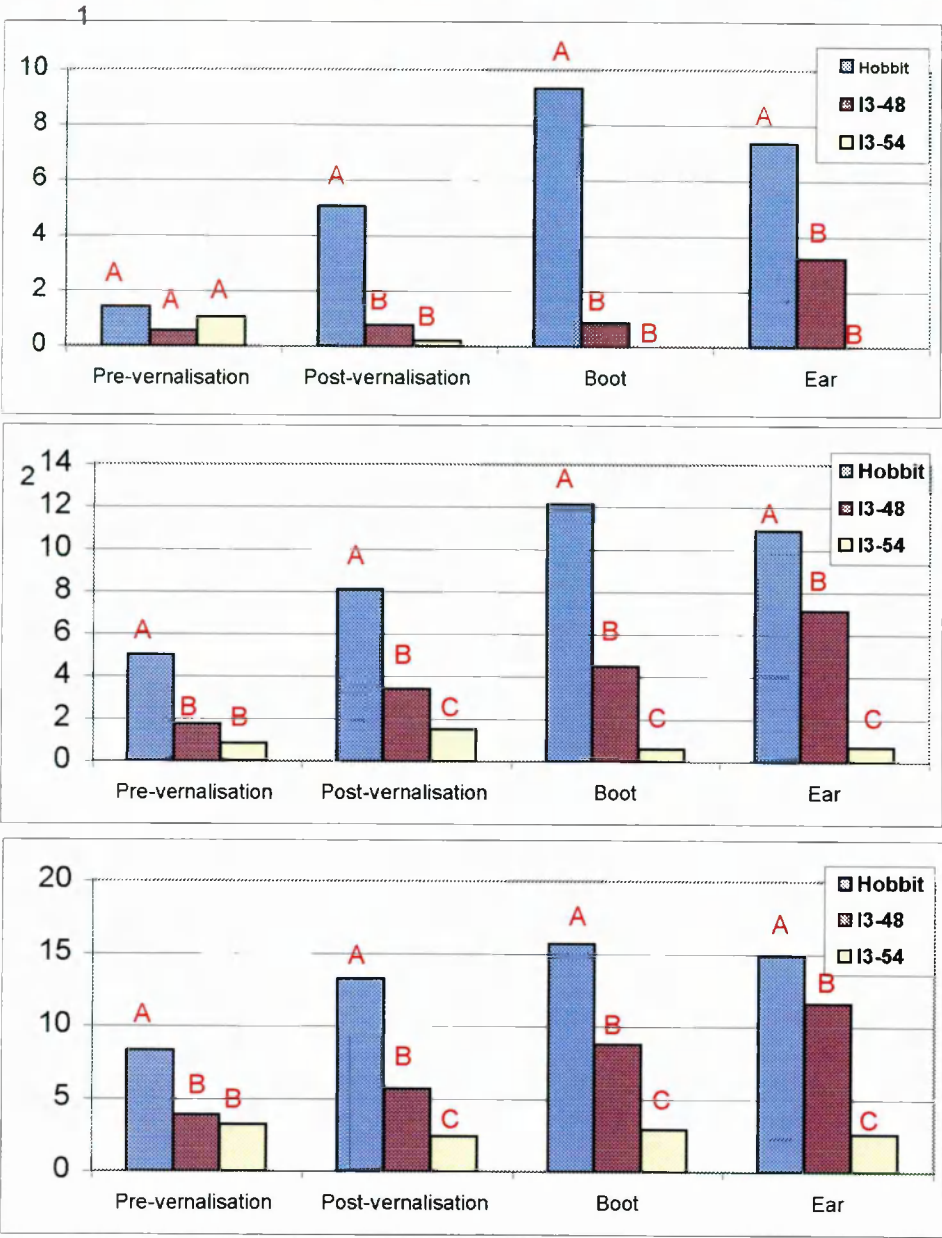
Genotypes with the same letters donot differ at $P=0.05$, comparisons to be made at the same growth stage only.
 For adult plant growth stages the percentage infection area on flag leaves were taken while the seedlings percentage infection was based on whole plant.

genotype, growth stage and genotype X growth stage, were significant for all leaves except for the youngest leaf which had non significant sum of squares for growth stage (Tables 2.3-2.5). Hobbit sib was found to be significantly more infected than the mutants on all three leaves at all growth stages (Fig. 2.2.b) except for pre- vernalised seedlings on the youngest leaf.

The F value for the growth stage variance was non-significant on the youngest leaves (Table 2.3), but I3-48 and I3-54 were significantly more resistant than 'Hobbit sib' at ear ($P= 0.05$) boot ($P= 0.01$), and post vernalisation ($P= 0.01$) stages for the youngest leaf (fig. 2.2.b1). For the other two leaves the F value for growth stage, variance was highly significant (Tables 2.4-2.5). All the three genotypes had significant differences in the infection levels at these three growth stages with Hobbit sib and I3-54 being the most susceptible and resistant genotypes respectively (Fig. 2.2.b2 and 2.2.b3). Also on comparing the overall disease levels between the three leaves (Fig. 2.2 b), all the genotypes at all four growth stages had less incidence of disease on the youngest leaf than on the middle and the oldest.

At the pre-vernalisation stage there was an overall lower incidence of disease on the seedlings of all three genotypes. At this growth stage non significant differences were detected on the youngest leaves of all the genotypes while for the other two leaves; the middle and the oldest, 'Hobbit sib' had significantly higher infection levels ($P= 0.01$) than the two mutants.

Fig. 2.2 (b) Powdery mildew infection levels on individual leaves at different growth stages.



X axis: Growth stages. Y axis: Mean infection

Panels 1, 2 and 3 represent mean percentage infection levels on the youngest (flag leaf for the adults while third in the seedlings), second (middle) and oldest (third from the top in adults and first in seedlings) leaves respectively of three wheat genotypes tested. Genotypes with same letters do not differ significantly at $P=0.05$, comparison to be made at same growth stage only.

Table. 2.3 Analysis of variance table of the powdery mildew scores on the youngest leaves (flag leaf for adults while third leaf in the seedlings) at different growth stages.

Source	Degrees of freedom	Mean Square	F Value	P
Genotype	2	84.98	21.06	0.0001
Replication	2	3.23	0.80	0.4621
Growth Stage	3	6.4	1.59	0.2214
Genotype X Growth Stage	6	22.13	5.49	0.0013
Error	22	4.03		

Table.2.4 Analysis of variance table of the powdery mildew scores on the middle leaf at different growth stages.

Source	Degrees of freedom	Mean Square	F Value	P
Genotype	2	201.46	409.71	0.0001
Replication	2	0.31	0.63	0.5407
Growth Stage	3	24.912	50.67	0.0001
Genotype X Growth Stage	6	10.55	21.47	0.0001
Error	22	0.49		

Table.2.5 Analysis of variance table of the powdery mildew scores on the oldest (third leaf from the top in adults and first leaf in seedlings) at different growth stages.

Source	Degrees of freedom	Mean Square	F Value	P
Genotype	2	321.42	95.75	0.0001
Replication	2	2.93	0.87	0.4321
Growth Stage	3	28.92	8.62	0.0006
Genotype X Growth Stage	6	10.13	3.02	0.0263
Error	22	3.35		

2.2.3 (a) Seedling resistance test I

All the seven mutants and the wild type were inoculated at three seedling stages to study the effect of the stage of plant development on resistance. In a previous experiment 2.2.2 (b), pre-vernalised seedlings of all the three genotypes had the same

amount of infection on their youngest leaves. One possible reason for this could be low incidence of disease, so this time a higher inoculum dosage was used (10.7 spores/mm² with an average spore viability of 52 %). As a result a significantly higher amount of disease on the second and third leaf stage seedlings was observed. Initially this experiment was designed to test the seedlings at three different growth stages, second, third and fourth leaf stages, scoring the second, third and fourth leaf individually at respective stages. Seedlings at the fourth leaf stage were chlorotic, also it was difficult to differentiate the fourth leaf individually as most of the plants were at the tillering stage. It was therefore, decided that only the second and third leaf stage seedlings would be scored.

Table. 2.6 Analysis of the powdery mildew scores on seedlings at second and third leaf stages.

Source	Degrees of freedom	Mean Square	F Value	P
Genotype	7	176.49	138.92	0.0001
Replication	2	23.93	18.84	0.0001
Growth Stage	1	1.81	1.43	0.2411
Genotype X Growth Stage	7	30.78	24.23	0.0001
Error	30	1.27		

The F value for genotype, growth stage X genotype and replication were highly significant. Overall ‘Hobbit sib’ had significantly more infection (P= 0.05) than all seven mutants and I3-54 was the most resistant. Similar results were observed at both growth stages Fig. 2.3 (a1-a2) but the resistance of I3-54 was more strongly expressed at the third leaf stage. I3-48 was significantly (P= 0.01) more resistant than the other mutants (apart from I3-54) at the third leaf stage seedlings only. The second leaves of I3-54 had significantly (P= 0.01) higher infections than the third leaves while a reverse trend was observed for I3-27 (P= 0.05).

Fig. 2.3 Powdery mildew infection levels at different seedling growth stages.



X axis: Genotypes. Y axis: Mean infection levels.

Panels a1 and a2 represent mean percentage infection levels on second and third leaves of the seedlings respectively in seedling resistance test 1 while b1, b2 and b3 mean percentage infection levels on first, second and third leaves of the seedlings respectively in seedling resistance test II.

Genotypes with same letteres do not differ significantly ($P = 0.05$)

The F values for replications were also observed to be significant, suggesting variation in disease incidence between replications. Replicate 2 had the highest mean disease score for all the genotypes at different growth stages. It also had the highest average spore density of 12.8 spores/mm² with a germination count of 52 %. The other two replicates had the average spore densities of 9.19 and 9.48 with germination counts of 54 % and 45 % respectively.

2.2.3 (b). Seedling resistance test II

Experiment 2.2.3 (a) was repeated with slight modifications, testing seedling resistance at first, second and third leaf stages and ensuring equal inoculum dosage for all three replications. Results from the analysis of variance Table. 2.7 for this experiment indicate highly significant F values for genotype, leaf stage and genotype X leaf stage. However this time F value for replications was non-significant, this shows the efficacy of the disease inoculation and development over all the three replications. An average spore density of 14.39 spores/mm² with a germination count of 45.6 % was recorded for this experiment.

Table. 2.7 Analysis of the powdery mildew scores on seedlings at different leaf stages.

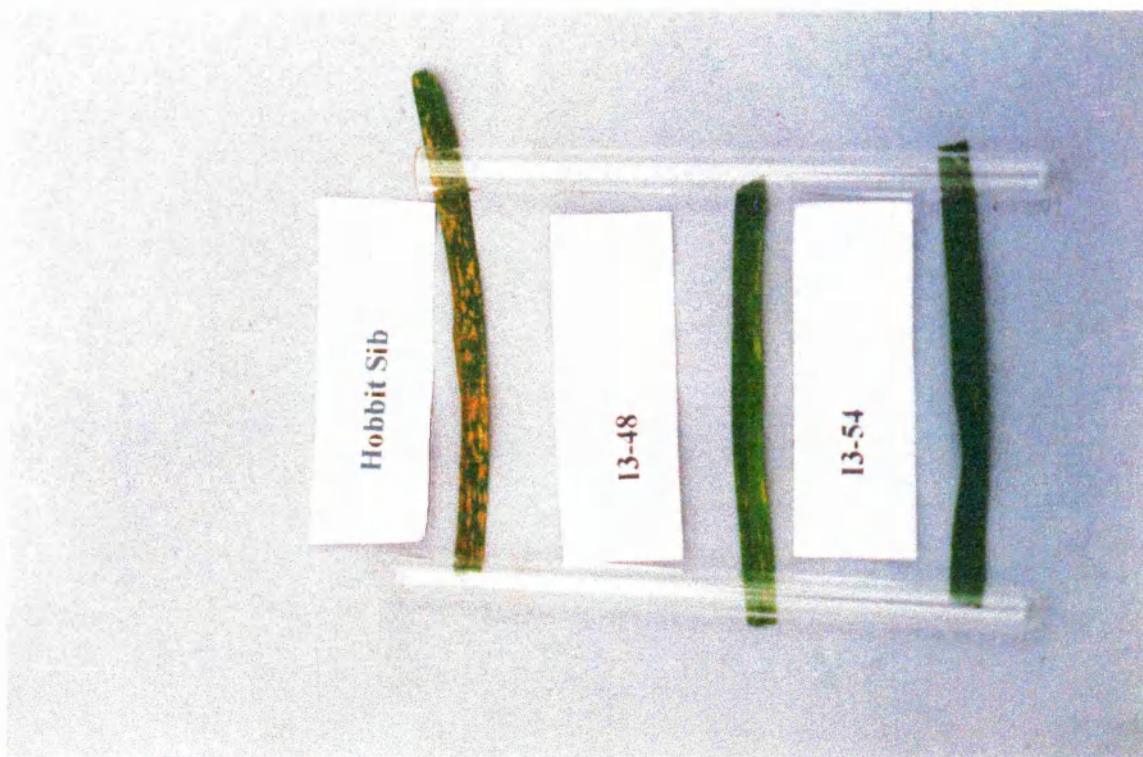
Source	Degrees of freedom	Mean Square	F Value	P
Genotype	7	638.44	68.86	0.0001
Replication	2	6.93	0.75	0.4788
Growth stage	2	123.21	13.29	0.0001
Genotype X leaf Stage	14	43.09	4.65	0.0001
Error	46	9.27		

Overall, the incidence of disease for this experiment was higher than the previous test on all leaf stages. I3-54 was significantly the most resistant mutant at all

the growth stages (Fig. 2.3.b1-b3), but resistance was more strongly expressed at the third leaf stage.

I3-48 was significantly more resistant than the wild type 'Hobbit sib' and most of the mutants at the first ($P = 0.05$) and third ($P = 0.01$) leaf stage (Fig. 2.4) but its mean scores were significantly higher than I3-54. All the other mutants were not significantly different from the wild type at all the three leaf stages.

Fig. 2.4. Powdery mildew infection on the third leaf of three wheat genotypes.



2.3 Discussion

A series of mildew tests was conducted during 1997-98 to investigate powdery mildew resistance in a Hobbit sib deletion series at both seedling and adult plant stages. All the mutants tested were more resistant than the wild type at the adult plant stage but I3-54 had significantly the highest resistance, followed by I3-48. Howie

(1997, Table 2.8) also reported I3-54 and I3-48 to be resistant in the limited tests he carried out. His results with other mutants were less consistent, although across two tests all showed some resistance.

Table. 2.8 Comparison between the APR tests for various mutants conducted by Howie (1997) and the present study.

Genotype	Trial 1	Trial 2	APR test
	Howie (1997)		
Hobbit Sib	75	50	50**
I3-21	90	30	10.8
I3-27	30	20	15.8
I3-32	50	7	10
I3-43	20	18	16.7
I3-48	1	2	5.1
I3-54	2	5	1.5*
I3-58	80	2	18.3

**** P = 0.01 * P = 0.05**

Both Howie (1997) and Worland and Law (1991) reported I3-48 and I3-54 deletion mutants to have high resistance to both yellow and brown rust. Howie found an association between a deletion on chromosome 4DL in I3-48 with this resistance. In the F2's of a cross between 'Rialto' and I3-48 he found this deletion to be co-segregating with resistance to rusts. However in the F3 he observed a few recombinants so he suggested this deletion to be closely linked with brown rust resistance while his results for yellow rust at F3 were inconclusive. The same deletion may also be associated with mildew resistance. Pink *et al.*, (1983) studying the aneuploid lines and chromosomal substitution lines of 'Chinese spring' particularly for chromosome 5 observed the resistance to yellow rust and mildew to be allelic.

The seedling disease tests revealed a developmental control in the regulation of resistance. The infection levels for the two resistant mutants i.e. I3-48 and I3-54 on first and second leaves were significantly ($P= 0.01$) higher than on the third leaves of

the seedlings (Fig 2.3). The first leaves of resistant barley genotypes were found to be more susceptible to mildew in the studies conducted by Wolters, *et al* (1993). Wolters *et al* identified mutants for the *mlo* locus in barley which undergo spontaneous formation of cell wall appositions (usually formed in fungal resistance reaction) under sterile conditions. A high degree of similarity was observed on comparing these spontaneously formed appositions with the infection triggered ones. They concluded a developmental genetic control in the formation of these appositions as these were detected only between 14-21 days old seedlings. At the same time a direct relationship between some necrotic and chlorotic lesions on the leaves with resistance was observed by them.

In the present study leaves at the third leaf stage of I3-54 were chlorotic during seedling disease tests. Initially it was thought to be because of under nourishment or lack of space (five seedlings per 7.5 cm pot). But on further testing all of these mutants in a disease free environment over five replications with two seedling densities of three and five per pot till two weeks after third leaf stage no leaf chlorosis was observed. So the leaf chlorosis could be because of host pathogen interaction, especially when the resistance is fully activated at the third leaf stage.

The results from the growth stage experiments indicated I3-48 and I3-54 to be significantly more resistant than wild type at all the five growth stages. Resistance at the adult plant stages was consistent with that in the APR test, however unlike in the APR test, disease scores for the two mutants were not significantly different from each other. This could be because of a reduction in the number of genotypes tested (2 degrees of freedom for the genotypes).

With ageing there seems to be a decline in the resistance of adults especially for I3-48 both across the growth stages and different leaves on the same plant at same growth stage (Fig. 2.2b). The flag leaf of I3-48 at the earing stage had more disease than the same leaf at the boot stage. Also the second and third leaves of both the mutants had more disease than the flag leaf though significantly I3-54 was less susceptible than I3-48. This could be because of a different resistance gene regulatory mechanism operative at different growth stages and for different leaves. Similar results were also observed by Carver and Adaigbe (1990) studying oat-mildew interaction, where they found less localised autofluorescent host cell response (HR, Papillae accompanied with phenolics) in the older leaves. Heitefuss *et al* (1997) also observed higher cumulative percentage of mildew infestation on the seventh and eighth leaf than on the ninth (Flag) leaf of a resistant barley genotype.

2.4 Conclusions

Two mutants, I3-54 and to a lesser extent, I3-48 are resistant to races of powdery mildew used in these tests under glasshouse conditions. The resistance in I3-54 is more strongly expressed from the third leaf stage of the seedlings and in adults; results for the moderately resistant I3-48 were variable although generally showed the same pattern.

The third leaves of the mutants I3-48 and I3-54 were significantly more resistant to powdery mildew than the wild type and the other mutants (Chapter.2). These mutants were neither immune nor exhibited hypersensitive reaction or necrotic specks upon inoculation or under disease free conditions as reported by Wolters *et al* (1993) and Greenberg *et al* (1994) on barley and *Arabidopsis* resistant mutants. The average disease scores on the third leaves of I3-54 and I3-48 were eight times and two and half times, respectively less than on Hobbit sib. To examine the resistance response in these two mutants fungal development was examined microscopically at different time intervals (12 hrs, 18hrs, 24 hrs and 72 hrs) after inoculation and compared with Hobbit sib.

3.1 Material and methods

The study was carried out in two independent tests one in summer of 1998 and the second in autumn of 1998, using the same plant material and methods. In the first test mildew development on the two mutants and Hobbit sib was compared at 12, 24 and 72 hours after inoculation (hai) while in the second at 12, 18 and 24 hai.

3.1.1 Plant material

Hobbit sib (susceptible), I3-48 (intermediate resistant) and I3-54 (resistant) were raised in the similar way as for seedling disease tests (section 2.1.3). The third leaves of all three genotypes were detached from the seedlings (when fourth leaf was fully emerged, GS 13.21, Zadoks *et al.*, 1974) and cut into 1cm long segments, excluding 1 cm from both the leaf tip and basal part. Twelve leaf segments of each genotype with their adaxial side uppermost were placed in a 6''X 4''X 1'' plastic box containing 30 ml of water agar (5gm/l) supplemented with benzimidazole (0.1 gm/l).

The experiment was laid out in a split plot design having three replications with one replication per box. Genotypes constituted the main plots and time periods after inoculation (12 hrs, 24 hrs and 72 hrs) were sub plots.

3.1.2 Inoculation

The mildew inoculum was collected from the ‘Cerro’, seedlings grown for disease resistance tests during 1998 (section, 2.1.2. (b) 2). Two series of differential tests were done, one for each part of the study and the same mildew virulence genes were identified. The isolate population had virulence for genes 1, 2, 3c, 3d, 4b, 5, 6, 8, 9, 11, *Mld* and *Mli* loci.

All three replications were inoculated separately by using a settling tower (45 cm high). Freshly produced mildew spores from ‘Cerro’ seedlings were collected on paper and were blown through a hole at the top of the settling tower which was placed over a box containing the leaf segments. After ten minutes the tower was removed from the top of the box. The box was covered immediately with its lid while the tower was washed with 96 % ethanol, to sterilise it. The tower was air dried briefly before placing it over the second box. The inoculation was done late in the evening and boxes were left in the glasshouse till the first sampling time (12 hai) interval. After sampling all the boxes were moved to the laboratory and incubated at a constant 20°C room temperature (ideal for mildew growth) for the rest of the test, as during the day time the temperature rises up quite sharply (> 20°C). Average spore density for each replication (10 counts) was calculated by counting the number of spores on the agar media between the leaf segments in a microfield view at 128 X magnification. Inoculum densities of 28.3 and 36.5 spores/mm² were observed for the first and second parts respectively.

3.1.3 Preparation of leaf segments for microscopic study

The four leaf segments per treatment were prepared for microscopic examination according to the method described by Boyd *et al* (1994, a). Segments were stained in 0.1% analine blue in lactoglycerol and 100 spores per leaf segment were scored using a modification of the scale described by Boyd *et al* 1994 (a) and Lyngkjear *et al* .,1997, Table 3.1.

Table 3.1. Scale used for recording different growth stages of *E.graminis*.

Developmental stage	Scale
Ungerminated	0
Germinated with a primary germ tube (PGT)	1
Appresorial germ tube (AGT)	2
Mature appresoria with a septum (MAP)	3
Infection peg (IP)	4
Secondary lobe	5
Tertiary lobe	6
Immature haustoria	7
Elongated secondary hyphae (ESH)	8
Colony	9

Most of these stages were clearly distinct at 100 X magnification but for some especially the infection peg and immature haustoria stages the magnification was increased to 400 X.

3.1.4 Statistical analysis

The mean value of the spore population (mildew growth on each genotype) for twelve hundred spores for each treatment was calculated but high standard errors of mean within each population were observed. Therefore the percentage germinated spores having MAP by 12 hai, IP by 18 hai and 24 hai and haustoria by 72 hai was calculated. These values were converted using arc sine transformations and then analysed using SAS statistical package.

3.2 Results

3.2.1. Distribution of spores, Test 1 (Fig 3.1a):

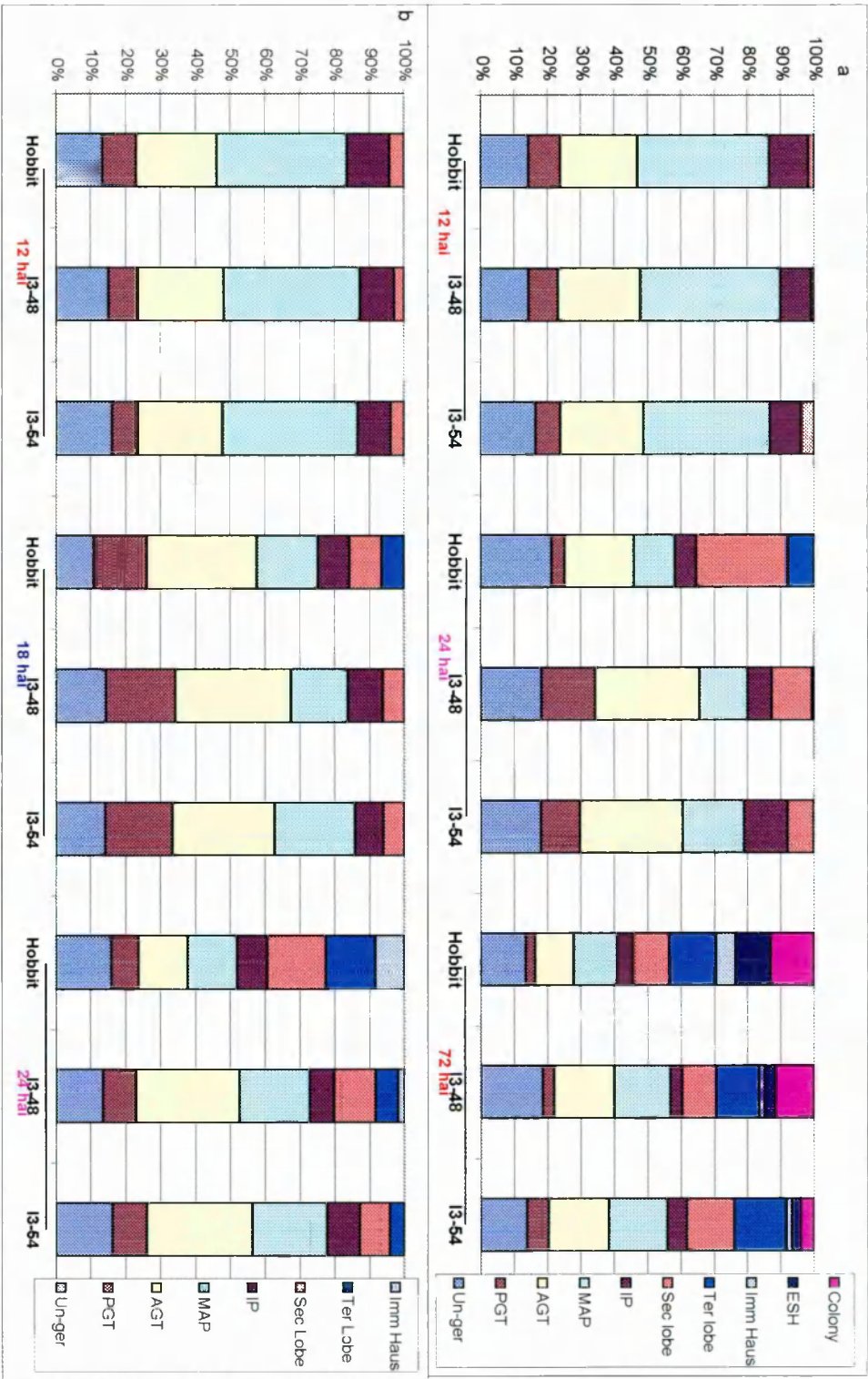
By 12 hai, germinated mildew spores showed same rate of growth on all the three genotypes with the majority of the viable spores having mature appressoria (MAP). A small proportion of them had also produced infection pegs, the first attempt by the mildew fungal hyphal to breach the cell surface. Papillae were detected beneath the PGT in the epidermal cells of all the three genotypes but they did not appear to inhibit mildew growth. More than 80 % of the spores on all the three genotypes had gone beyond the PGT stage. Almost the same proportion of ungerminated spores were detected on all the three genotypes.

Differences in fungal growth on the resistant and susceptible genotypes occurred between 12-24 hrs. On the susceptible genotype, Hobbit sib, there were comparatively more spores having secondary and tertiary lobes than on the two resistant mutants (five times and three times more than I3-54 and I3-48 respectively). Very few haloes or papillae response were observed on all the three genotypes. In Hobbit sib more than 78 % of the total germinated spores with mature appressoria produced infection pegs and subsequent growth stages, whilst for I3-48 and I3-54 it was 57 % and 53 % respectively. The percentage of germinated spores with at least infection pegs was 53 % for Hobbit sib but 24.4 % for I3-48 and 25.8 % for I3-54.

At 72 hai the percentage of spores successful in producing haustoria compared with the total number of spores having appressoria was three times more in Hobbit (34.6 %) than I3-54 (10.6 %) while for I3-48 this figure was 21.3 %.

As the time interval between the different fungal growth stages assessed was unlikely to be constant so the results were expressed in relation to the number of

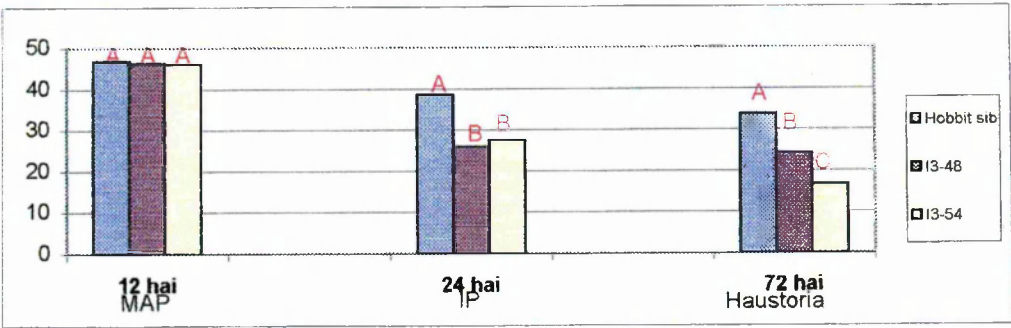
Fig. 3.1 Percentile distribution of mildew spores at different growth stages on three wheat genotypes in two experiments: (a) 12, 24 and 72 hours after inoculation, (b) 12, 18 and 24 hours after inoculation.



germinated spores at a particular growth stage at a particular time. Hence the number of spores forming mature appressoria by 12 hai, producing penetration peg or subsequent stages by 24 hai, having haustoria or subsequent stages by 72 hai was expressed as a percentage of the total number of germinated spores for that variety. These percentages were transformed using arc sine transformations for calculating the analysis of variance for each time interval separately.

There was no difference in fungal development on the three genotypes by 12 hai (Fig. 3.2a). However, by 24 hai, fungal development was inhibited in the two mutants in comparison to Hobbit sib (IP formation) (LSD= 4.89, P= 0.05). By 72 hai, fungal hyphae were inside the cell and the resistance of I3-54 to haustorial development was significantly higher than that of I3-48 (72 hai) (LSD= 1.41, P= 0.05).

Fig. 3.2a. Percentage of spores (transformed data) reaching a designate stage of development as a proportion of total germinated spores.



X axis : Genotypes X time intervals after inoculation. Y axis: Percentage (transformed) of germinated spores with mature appresoria (MAP) 12hai, infection peg (IP) 24 hai and haustoria 72 hai. Genotypes with same letters not different at P= 0.05, comparison to be made at the same interval only.

3.2.2 Distribution of spores, Test II (Fig. 3.1 b):

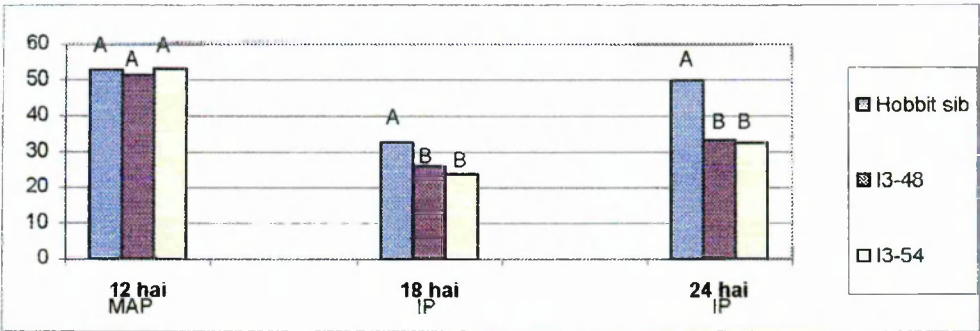
To check the consistency of results from the first test and to study more closely the fungal development during the first 24 hai, the mildew germlings were examined at 12, 18 and 24 hai. At 12 hai fungal development closely mirrored that

seen in the test I. However, at 24 hai germlings were developing somewhat faster than in test I.

Almost the same rate of growth was observed on all the three genotypes at 12 hai. By 18 hai germinated spores on Hobbit sib had a faster rate of growth than the two mutants with a significantly higher percentage of spores at the infection peg stage or beyond. On Hobbit sib, 6.5 % of the germinated spores had tertiary lobes while this stage was totally absent on the two mutants. Spores on Hobbit sib maintained a faster rate of growth at 24 hai with 8.4 % producing haustoria while only 1.3 % on I3-48 were at this stage, and none of the spores on I3-54 had reached the haustorial stage. A significantly higher percentage of the spores with IP or later growth stages to the ones with MAP were detected on Hobbit sib (74 %) than on I3-48 (54 %) and I3-54 (52 %).

Similar results were obtained for the analysis of variance (ANOVA), with resistance in the two mutants being expressed by 18 hai (LSD= 2.61, P= 0.05) and continuing at 24 hai (LSD = 3.91, P= 0.05) Fig. 3.2b. This indicates the consistency of the results over the two parts of the experiment. Significant differences in fungal penetration between the wild type and the two mutants were again observed at 18 hai, and were greater at 24 hai.

Fig. 3.2b. Percentage of spores (transformed data) reaching a designate stage of development as a proportion of total germinated spores.



X axis : Genotypes X time intervals after inoculation. Y axis: Percentage (transformed) of germinated spores with mature appresoria (MAP) 12hai, infection peg (IP) 18 and 24 hai. Genotypes with same letters not different at P= 0.05, comparison to be made at the same interval only.

3.3 Discussion

Plants protect themselves against pathogens by the production of various chemical and physical barriers to restrict their growth at specific stages. The production of all these substances is host specific and determined genetically. Some of these compounds could either be present constitutively on the plant surface (externally) hindering pathogen growth *in situ* (Knogge, 1996, Osbourn 1996) or internally (Bowling *et al.*, 1994, Wolter *et al* 1993) restriction at the penetration stage or their production could be triggered in the plant system upon pathogen entry inside the cell (Bowles, 1990, Heath, 1995).

In the present study differences in the development of germinating mildew spores on the leaves of resistant mutants (I3-48 and I3-54) compared with the wild type (Hobbit sib) could not be detected until the IP formation, that is to say at the first penetration attempt. This indicates that the seedling leaf surface of the two resistant mutants does not present a more significant challenge than the wild type. Significantly less spores were detected at the secondary lobe stage 24 hai on the two mutants than the wild type. There was no evidence of HR and a significant number of spores were able to form haustoria in all the three genotypes. However the percentage number of germinating spores producing haustoria was much lower on I3-54 (9.3 %). This would still be sufficient to establish a high level of infection on the leaves if there was no further barrier to fungal development. From the disease tests it is known that the disease levels on I3-54 were much lower than on Hobbit sib, so the inference is that the haustorial development is impeded in the mutants, either as a result of haustorial collapse or restricted digits formation (Niks, 1983, Niks, 1986, Niks and Dekens, 1991). This would lead to weak sporulation and finally lower

disease as observed in the disease tests. At the same time comparison of the mildew development on the two mutants at 72 hai (Fig.3.2a) indicated significant differences between them suggesting different levels of post-haustorial resistance for each mutant. This was further supported by the results from the disease tests as significant differences in the infection levels of these two mutants on the third leaves were observed (section. 2.2.3). Experiments would need to be run over a longer period to test for post-haustorial resistance.

Chapter 4

Molecular studies

Disease and histological studies (Chapters 2 & 3) on the third leaf of seedlings of the two resistant mutants and wild type 'Hobbit sib' indicated significant differences between genotypes for mildew resistance. One way in which plants protect themselves against potential pathogens is by the production of various compounds which could prove inhibitory to pathogen growth. Some of these compounds might be present constitutively or their production might be triggered during pathogen infection (Bowling *et al.*, 1994, Heath, 1995). An example is PR proteins which are associated with resistance gene response (Davidson *et al.*, 1987, Davidson *et al.*, 1988, Boyd *et al.*, 1992, Boyd *et al.*, 1995). To determine whether expression of PR proteins was associated with the resistance of Hobbit sib deletion mutants, total RNA from the mutants and wild type grown under disease free conditions was extracted and probe hybridised with cDNA clones of various PR proteins.

4.1 Material and methods

The work for this study was carried out as two independent tests, using the same plant genotypes and experimental methods. To determine the association of PR proteins with mildew resistance more precisely, the plant material for each RNA test was grown alongside the two seedling resistance tests under the same environmental conditions (2.1.3a).

4.1.1 Plant Material

All seven mutants (I3-21, I3-27, I3-32, I3-43, I3-48, I3-54 and I3-58) and Hobbit sib, laid out in a randomised block design (RBD) with 3 replications, were raised to the third leaf stage. Each treatment consisted of 10 seedlings in two 7.5 cm pots i.e. 5 seedlings per pot. The plant material for the seedling disease tests was

moved to another glasshouse and inoculated while the third leaves of these 10 seedlings per treatment were harvested on the same (inoculation) day. Leaf tissue was harvested and immediately frozen in liquid nitrogen prior to storage at -70°C .

4.1.2 RNA extraction

RNA was extracted from the stored leaf tissues of all the mutants and wild type by taking one replication at one time. The method used was essentially similar to the method developed by Chomczski and Sacchi (1987). Plant tissue was moved to a polystyrene box containing liquid nitrogen (N_2) and was then homogenised using a Mikro-Dismembrator. The material was ground for 2 minutes and the powdered leaf tissue was transferred to Falcon centrifuge tubes kept in liquid N_2 . One gm of the frozen homogenised tissue was put into a screw capped corex tube and 10 ml of trizol reagent (GibcoBRL, Life Technologies) was added. After gentle shaking the sample was left for 5 minutes at room temperature. Two ml of chloroform was added and the tube was capped securely again. Samples were shaken vigorously for 15 seconds and left at room temperature for 2-3 minutes. The tubes were then centrifuged at $12000 \times g$ for 15 min at $2-8^{\circ}\text{C}$. Following centrifugation of the mixture an upper colourless aqueous phase was transferred to a fresh 15 ml corex tube and an equal volume of isopropyl alcohol was added for RNA precipitation. Samples were incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at $12000 \times g$ at $2-8^{\circ}\text{C}$. The supernatant was removed and the RNA pellet washed with 10 ml of 75 % ethanol by vortexing for a short while and centrifuging for 5 min at $7500 \times g$. The RNA pellets were air dried for 20-30 min. 100 μl of distilled H_2O was added and vortexed for 1 min. The corex tube was capped with parafilm and stored at -20°C for two hours. After a gentle vortex the contents were transferred to a sterile eppendorf tube.

4.1.3 RNA quantitation

RNA was quantitated by using the spectrophotometer reading at 260 nm.

$\text{RNA } \mu\text{g/ml} = A_{260} \times 40 \times \text{dilution factor}$

A_{260} = absorbance (in optical density) at 260 nm

40 = extinction coefficient of RNA

4.1.4 RNA gel electrophoresis

To check the quality of the RNA sample a 1 % agarose (formaldehyde) gel with 10 μg of each RNA sample was electrophoresed overnight at low voltage (10-15 volts). Gels were prepared and RNA samples were analysed using the following method.

4.1.4.1 Gel preparation:

1-1.5 gm of agarose + 73 ml of H_2O + 10X MOPS was heated for 2 minutes in the microwave to dissolve the agarose and allowed to cool down. After this 17 ml of formaldehyde (37 % v/v solution) was added and the contents were poured immediately into a gel tray in a fumehood.

4.1.4.2 Preparation of RNA sample and loading:

Ten μg of RNA in a final volume of 3 μl was denatured by the addition of 6.5 μl deionized formamide, 1.25 μl of 10X MOPS and 2 μl of formaldehyde (37 %) and was incubated at 65°C for 5 minutes. Immediately after this it was chilled on ice and 2.5 μl of 50 % (v/v) glycerol, containing 0.1 mg/ml bromophenol blue was added. The RNA sample was loaded on the formaldehyde/agarose gel and was run for 16-18 hrs at 10-15 volts.

4.1.4.3 Quality of RNA:

After 16-18 hrs the gel was stained with 5 µl ethidium bromide (10 mg/l) in 200 ml of H₂O for 30 minutes and then washed for 2 hrs in distilled H₂O and photographed under UV light. The sharpness of the bands (25S and 18S) indicated the quality of RNA of each sample. If the bands were smeary RNA was re-extracted. At the same time it also gave an idea of the quantity of total RNA sample loaded or present for each sample. If the loadings were not equal the RNA was re-quantitated.

4.1.5 Northern Blotting

Twenty µg of RNA from each genotype, 6 µl in total volume (doubling all the contents of RNA sample preparation, Section 4.1.4.2) was loaded on 1 % agarose gel and run overnight 10-15 volts. 4 µg of RNA millenium marker (Ambion, RNA Inc, Company, USA) was also loaded on the gel along with the test RNA samples. All efforts were made to have equal loadings in each track. A fluorescent ruler was placed next to the marker track and its picture was taken under UV light. This helped to determine the sizes of the transcripts present on the Northern blot after hybridisation. The marker track and the wells from the gel were excised before blotting.

The gel was then transferred to a nylon membrane (Hybond-N; Amersham International, UK) with 20X SSC as a transfer buffer (capillary action) according to the procedure described by Farrell (1993). The following day the nylon membrane was taken off and air dried for approximately one hour. Later it was exposed for 2 minutes under the UV light, which results in the activation of nitrogenous bases and forms covalent bonds with the surface amines and fixes the RNA to the membrane. The membranes were stored at -20°C after wrapping in saranwrap until used.

4.1.6 Hybridisation and Probing

Northern blots were prehybridised in 15 ml/filter 1X Denhardt's III, 1X HSB, 0.5 g/l salmon sperm DNA (denatured by boiling for 5 minutes immediately prior to use). Filters were sandwiched between two nylon meshes (HYBAID) and were put in a 35 mm diameter hybridisation tubes. Tubes were placed in a rotisserie incubator (Hybaid) for 4 hrs at 65°C. These were then probed with various cDNAs encoding PR proteins from cereals (Table. 4.1).

Table. 4.1 List of cereal cDNA probes/inserts (PR proteins), used for hybridisation and crop species from which they were isolated and source.

Name	Function	Crop species	Source
pHv PR1b	PR1	Barley	Brynegelsson <i>et al.</i> , 1994
pW1R232	Thaumatococcus like	Wheat	Rebmann <i>et al.</i> , 1991 (a)
pPDX381	Peroxidase	Wheat	Rebmann <i>et al.</i> , 1991 (b)
pBH6-903	Oxalate oxidase	Barley	Zhou <i>et al.</i> , 1998

The cDNA inserts were diluted to a concentration of 25 ng in 45 µl volume of distilled H₂O and were denatured in boiling water bath for 2-3 min and were transferred to a Rediprime labelling mix tube (Amersham, UK) containing dATP, dGTP, dTTP, exonuclease free klenow enzyme and random primers. To this, 5 µl [α^{32} P]-dCTP (3000 Ci/mmol) was added and incubated at 37 °C for > 10 mins. The reaction was stopped by adding 20 µl of column dye and 120 µl of column buffer. The probe was cleaned using a sephadex column to eliminate non incorporated dNTP's.

4.1.7. Sephadex column

A column tip was prepared with some glass wool gently packed down to the tip of the column. 1 ml of sephadex 50 in TE buffer was filled to the column tip. The column tip was placed in a clamp. After sometime the column was again topped up to the ridge with TE buffer. To this the whole volume of the radioactive sample was

added. The fractions were collected in the clean eppendorf tubes. When the column stopped dripping, the column was moved over another eppendorf and 200 µl of column buffer was added. The same procedure was repeated for 8 eppendorfs. The column was lowered in the 9th eppendorf and discarded. The radioactive counts per eppendorf were measured using a radioactive mini monitor and the contents of two hottest one's were mixed (400 µl) while others were discarded. To this fractionised radioactive probe (400 µl) 40 µl of 3M NaOH was added to denature it and after 5 minutes the entire contents were shifted to the hybridising tubes and kept overnight at 65 °C in the orbital shaker.

4.1.8 Washing of the filters

After 16 hrs of hybridisation, the hybridisation solution was removed from the tube and discarded under running tap water. The filters were washed for 10 minutes in 50 ml of 6X SSC, 0.1 % SDS (pre-equilibrated at 65 °C). This was to reduce background and overall radioactivity of the blots before removing the blots from the hybridisation tubes. Filters were then transferred from the tubes into 220 mm X 220 mm polycarbonate boxes (Stewart Plastics) containing 2X SSC, 0.1 % SDS and washed for 15 min at room temperature with gentle shaking. This was followed by another 15 minutes wash in 0.1X SSPE, 0.1 % SDS. Where necessary another wash at 65 °C in 0.1X SSPE, 0.1 % SDS was carried out to reduce the counts on the filters. Filters were generally washed until the counts on the filters were between 10-20 cps.

4.1.9 Autoradiography

Washed filters were dried briefly on 3MM paper and were wrapped in saranwrap. These were then exposed to X-ray film (Kodak biomax MS film) placed in a Biomax intensifier screen, arranged with the film between the screen and filter. The

arrangement was placed in a side lock autoradiography cassette at -70°C for various lengths of time. X-ray film was developed in an automatic film developer (Fuji X-ray Film Processor, RGII)

4.1.10 Quantitation of the northern blots

Results from the northern blots were quantitated by using a phosphorimager (Molecular dynamics) according to the procedure described by Johnston *et al.*, 1990 and the loading for each genotype was standardised by reprobing all the blots (after stripping, section 4.1.10) with a 9 kbp, EcoRI fragment of wheat 18-25S rRNA repeat, clone pTA71. The ratio between the phosphorimager reading for rRNA and PR protein for the same sample was calculated to determine the level of expression for that PR protein in the sample genotype.

4.1.11 Stripping of northern Blots

The 0.1X SSPE + 0.1 % SDS solution was first preheated to 95°C and poured in a sandwich box containing the filters. The sandwich box was then incubated for 10 minutes or more in a shaker at 65°C . This procedure was repeated until no counts were detectable on the filters.

4.1.12 Transformation of *E. coli* competent cells

Various plasmids containing cDNA inserts listed in Table. 4.1 were transformed into *E.coli* DH 5 α^{TM} competent cells from GIBCO BRL, Life Technologies, UK. 0.5 μl (100 ng) of the DNA insert was mixed with 10 μl of *E. coli* competent cells and incubated on ice. After 30 min of incubation on ice the contents of the eppendorf (Insert + *E.coli*) were given a heat shock for 45 sec at 42°C . They were then again incubated on ice for 2 min. After this 90 μl of SOC, Life technologies medium (cell growth media) were added and the eppendorf was shaken for 1 hr at

37°C at 225 rpm. After this the cells were plated on L.B agar plates containing a 1000X dilution of ampicillin (100 mg/ml stock solution) and a 400X dilution of Xgal (2 % stock solution) as a selection marker and were incubated overnight at 37 °C. The following day the plates were transferred to 4 °C. A single colony was streaked out on to a new plate and grown overnight again at 37 °C.

4.1.13 Mini and Maxi Preps

For mini prep an overnight culture of 2 ml L. broth, 2 µl of ampicillin (100 mg/ml) and single colony of the transformed *E. coli* was set in a universal tube and grown at 37°C in a shaker at 225 rpm. The contents of the universal were minipreped using a Promega mini prep kit. For a maxi prep an overnight culture of 400 ml L. broth, 400 µl of ampicillin (100 mg/ml) and single colony of the transformed *E. coli* was set in a flask with ridges and grown at 37°C in a shaker at 225 rpm. The contents of the flask were maxipreped using a Qiagen maxi prep kit. The isolated DNA pellets were redissolved in distilled H₂O and quantitated using a spectrophotometer.

4.1.14 Plasmid DNA Quantitation

The plasmid DNA was quantitated using the spectrophotometer reading at 260 nm and was stored at -20°C.

DNA mg/ml = A₂₆₀ X 50 X Dilution factor

A₂₆₀ = Absorbance (in optical densities) at 260 nm

50 = extinction coefficient of DNA

4.1.15 Digestion of plasmids with restriction enzymes

The isolated plasmid DNA was digested with specific restriction enzymes (Table. 4.2) to release the insert. One µg of plasmid DNA was digested in a final volume of 15 µl, containing 1.5 µl of 10X restriction digest buffer, 1.5 µl of the

enzyme (10 units/ μ l) at 37 °C for an hour. The reaction was stopped using 5 μ l of 6X loading buffer. Samples were stored at -20 °C until used.

4.1.16 Electrophoresis of the restriction digests

The digested plasmid DNA was electrophoresed for 2-3 hrs at 60 volts in a 1 % agarose gel in 1X TAE buffer. The size of the insert was determined by comparing with a kilobase DNA ladder (Pharmacia Biotech lab). After making sure that the insert was of right size, a 50 μ l restriction digest containing 10 μ g plasmid DNA insert was electrophoresed in a low melting point 1 % agarose gel in TAE buffer. The concentration of the enzyme (5 μ l) and the restriction digest buffer (5 μ l) were also increased accordingly. The electrophoresis was carried out for 2-3 hrs at 60 volts. Using a clean scalpel blade the desired cDNA insert band was excised and transferred to an eppendorf. It was later gel purified using promega gel purification kit according to manufacturer's instructions.

4.1.17 Quantitation of the insert cDNA by dot spot analysis

Five μ l of DNA samples of known concentration were mixed with 5 μ l of 2 ng/ μ l of ethidium bromide and placed on a petridish. Aliquots of the gel purified DNA fragments to be quantitated, were also mixed with 2 ng/ μ l of ethidium bromide. The petri dish was viewed and photographed under UV light and the concentration of the cDNA insert was determined by comparing the intensity of their dot spots with that of standard DNA samples. The purified cDNA insert after quantitation was stored at -20 °C and used for probing the northern blots.

Table.4.2 Enzymes used in digestion of the various plasmids for isolating the cDNA inserts.

Name	Plasmid/Vector	Enzymes	Size of insert (bp)
pHvPR1b	pBlue script	EcoR1	759
pWIR 232	pBlue script	EcoR1	670
pPOX 381	pBlue script	EcoR1	1247
pBH6-903	pBlue script	Bam HI-HindIII	904
rRNA	PTA71	EcoR1	9000

4.1.18 Stock solutions

1. 6 X loading buffer15% ficol type 400, 0.025% bromophenol blue, 0.1M EDTA. Stored at -20°C.
2. TAE40 mM Tris Base, 20 mm Acetic acid, 2mM EDTA; pH 8.0.
3. 20X SSC3M sodium chloride, 0.3 sodium citrate
4. 10 X Denhardts III2%gelatine, 2% Ficoll-400, 2% PVP-360, 5% sodium pyrophosphate, 6% SDS: leaving stirring overnight to dissolve. Stored at -20°C.
5. 5 X HSB stock3M Nacl, 0.1M PIPES, .2mM EDTA; pH 6.8.
6. Salmon sperm DNA5g in 1 litre; dissolve with stirring. Store -20°C.
7. 20 X SSPE3M sodium chloride, 0.2 sodium dihydrogen orthophosphate, 0.02 M EDTA.
8. L.Broth20 g per litre of LB Broth Base (GIBCO-BRL).
9. X gal2 % w/v 5-Bromo-4-Chloro-3-Indoyl-β-D galactoside in n,n-Dimethyl formamide (DMF); Stored at -20°C.
10. MS 1%1% sucrose w/v in distilled water, 0.9% w/v of agar. Autoclaved and stored at 5°C.
11. 10X MOPS0.4 M MOPS, 1 M sodium acetate, 0.02 M EDTA dissolved in 1 litre of distilled water. pH 7. Stored at 5°C in dark.
12. RNA loading dye50% glycerol, 1mM EDTA (pH 8.0), 0.25% bromophenol blue, 0.25 % xylene cyanol FF

13. SOC medium 10 ml	Tryptone 2%, Yeast 0.5 %. NaCl 10mM, KCl 2.5mM, MgSO ₄ 10mM, MgCl ₂ 10mM and Glucose 20mM.
14. Column dye	20 ml TNE, 0.1 mg Blue Dextran 3000, 0.1 mg Orange dye.
15. Column buffer	25 ml TNE 10X, 2.5 ml 10 % SDS, 222.5 ml H ₂ O.
16. Sephadex G-50	100 ml Column buffer, 5 gm of sephadex G-50, store at 4 °C.
17. TNE	20mM TRIS, 20mM NaCl, 1mM EDTA in 5 l, pH 8.0.
18. TE	TRIS 121.1 gm, EDTA 37.2 gm, 800 ml H ₂ O, pH 8.0

4.2 Results

4.2.1 Standardisation of northern blots

A series of northern blots was prepared to determine the expression levels of various PR proteins (mRNA) in the Hobbit sib deletion series mutants. The expression levels could vary according to the amount of the total RNA loaded for each sample on the northern blot. So the RNA loadings on all the northern blots were standardised using a wheat 9 kb, 18-25S rRNA, DNA probe from wheat (Gerlach and Bedbrook, 1979). The phosphoimager value for the 25S band for each sample genotype was taken.

4.2.2 PR protein expression studies: Test I

4.2.2.1 Wheat Thaumatin Like Protein (mRNA) expression studies

Rebmann *et al.*, (1991a) found mRNA hybridising to a thaumatin like (TL) protein's cDNA sequence accumulated rapidly in wheat leaves upon inoculation with *E. graminis* f.sp. *hordei*. Subsequently, when these plants were challenged with *E. graminis* f.sp. *tritici* they showed a high level of resistance indicating that challenge with a non-host pathogen induced resistance to a host pathogen. To determine the

expression levels of this PR protein in the Hobbit sib deletion series, three northern blots (replications) having RNA samples from all the eight genotypes were probe hybridised with the cDNA of wheat TL protein. Results indicated that a transcript of ~ 800 bp hybridising to this cDNA probe was observed consistently in three mutants (I3-27, I3-48 and I3-54) over all three replications (Fig. 4.1.1c-3c).

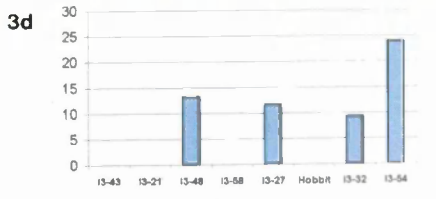
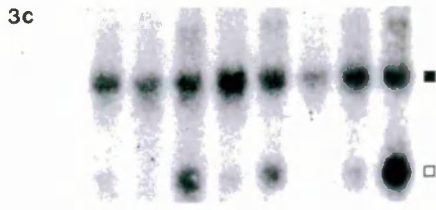
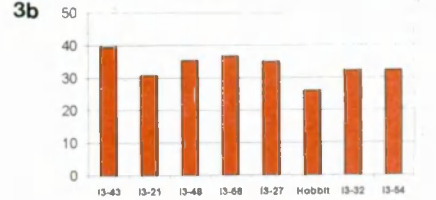
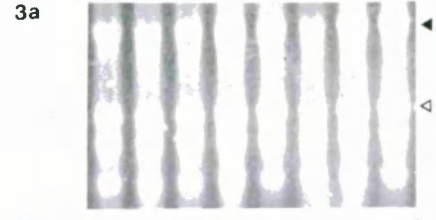
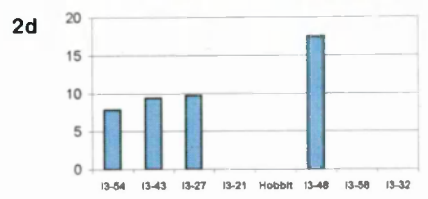
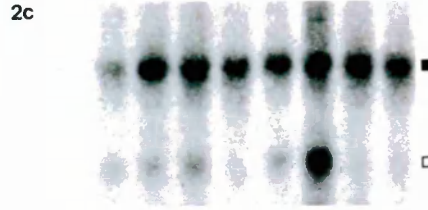
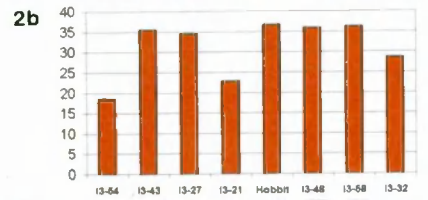
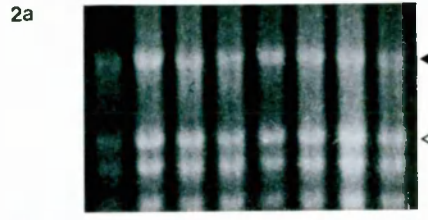
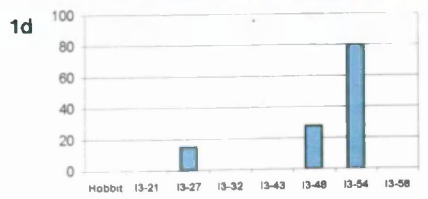
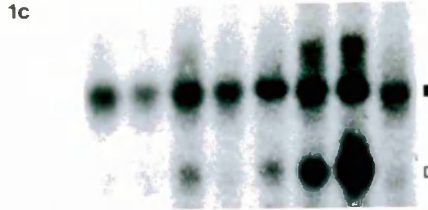
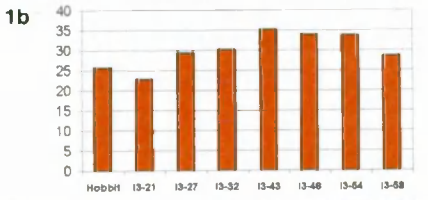
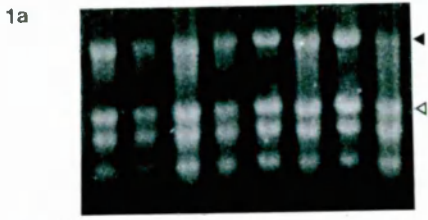
The level of expression of the ~ 800 bp band in Hobbit sib varied between different replicates; a weak transcript band was detected in replication 2 but no bands were detected in replicates 1 and 3. A weak transcript band was also present in I3-32, I3-43, and I3-58 in some of the replicates. These faint bands can be seen in the autoradiographs (Fig. 4.1.1c-3c) but were not always detected by the phosphoimager machine during the quantitation procedure. The reason for this was unclear but it might be that shorter exposure times were used for the phosphoimaging relative to autoradiograph exposures. This might have resulted in lack of detection of these weaker bands against the high background signal.

In replicate 1 there was approximately equal loading of RNA from Hobbit sib and mutants I3-27, I3-48 and I3-54 as judged by the intensity of rRNA staining in the ethidium bromide stained gel (Fig. 4.1.1a). However, the level of expression of the 800 bp band in the three mutants was considerably higher than in Hobbit sib indicating significant induction of expression. The interpretation of the data from the other two replicates was more difficult because of variation in loadings. However, it is possible to conclude that there was induction of the 800 bp band in I3-48 compared to Hobbit sib control in replicate 2.

The blots were then probed with rRNA and the phosphoimager values (ratio) for the expression levels of TL to 25S was calculated for three mutants, I3-27, I3-48

Fig. 4.1. Expression pattern of thaumatin like (TL) in the three northern blots, test I, one for each replication.

Panels 1, 2, 3 represent the three replications while a, b, c and d represent the ethidium bromide stained northern gel, phosphorimager quantitative value for 25S (▲), autoradiograph after hybridisation with TL cDNA and phosphorimager quantitative value for the actual transcript (800 bp, □). Symbols ■ and ◁ represents the higher molecular weight transcript (1.7 kb) and 18S rRNA band respectively. All the three northern blots were washed for 10 minutes each with 6X SSC, 2X SSC and 0.1X SSPE at room temperature. Following these three washes northern blot for replication 1 and the other two for replication 2 and 3 were washed with 0.1X SSPE at 65°C for 15 and 10 minutes respectively. All the three northern blots were exposed for 48 hours to a maximum sensitivity X-ray Kodak film.



and I3-54 (Table. 4.3). The level of expression of TL in mutant lines I3-27, I3-48 and I3-54 appeared to be higher than that in Hobbit sib in all the three replications. However, the relative level of induction compared to the rRNA control varied between replications. For example, in replication 1 the induction of TL in I3-54 was much higher than that in I3-27 or I3-48 but in replication 2, the level of induction in I3-54 was similar to that in I3-48.

Table 4.3. Ratio of the expression value of TL to 25S for three mutants in three replications.

Genotypes	Replication1	Replication 2	Replication 3
I3-27	0.49	0.28	0.33
I3-48	0.81	0.48	0.37
I3-54	2.3	0.43	0.74

Comparisons to be made within a replication not between replications.

A second transcript of ~ 1700 bp was also detected, Fig. 4.1.(1c-3c). According to Rebmann *et al.*, (1991a) the probe should hybridise to a mRNA of ~ 800 bp. So the lower molecular band (~ 800 bp) detected in some mutants was the actual mRNA transcript of the TL protein while the higher molecular weight of 1700 bp could be either the primary transcript (unprocessed RNA) or there could be another homologous gene (transcript).

4.2.2.2 PR-1 expression studies

PR-1 has been reported by several workers as a close indicator of SAR (Bowling *et al.*, 1994 and Dietrich *et al.*, 1994). So to test the expression of PR-1 protein in the Hobbit sib mutants, a northern blot was hybridised with a cDNA encoding PR-1 from barley. The northern blot had RNA samples from I3-48, I3-54 and Hobbit sib, over three replications. From the disease tests Chapter.2 these two mutants were significantly more resistant than the others. So it was decided to restrict the molecular study to only three genotypes. The advantage of this northern blot over

the previous one was that all RNA samples from the three replications would be processed at the same time for running on the same gel and blotting. Along with these nine RNA samples, three more RNA samples from *in vitro* raised pre vernalised seedlings at GS 13.21 Zadoks *et al* (1974) (grown as described in Section 2.1.1.1) of I3-48, I3-54 and Hobbit sib were also loaded as a positive control for the PR-1 protein expression. In previous studies on the *in vitro* raised seedling material the expression of PR-1 gene was detected (data not shown). The northern with twelve RNA samples was probe hybridised with a barley cDNA (759 bp) encoding PR1 protein (Brynegelsson *et al.*, 1994). They observed a 900 bp transcript in 11 days old barley leaves 4 hrs after inoculation with powdery mildew. In the present study also a similar size (900 bp) of transcript was detected (Fig 4.2.1c-4c) in the glass house raised mutants (I3-48 and I3-54) and in all the three RNA samples of *in vitro* material.

The ratio of PR1 to 25S value was quite variable among genotypes and replications (Table. 4.4). As no PR-1 expression was detected in glasshouse raised ‘Hobbit sib’ and I3-48-replication 3, no standardised expression value (ratio) for PR-1 could be determined. A strong induction of PR-1 was observed in the *in vitro* raised plant material for all the three genotypes. However the expression levels in the two mutants were 3-5 times higher than in the wild type.

A higher molecular weight transcript of ~2.0 kb (Fig.4.2.4c) was also observed in the RNA samples from the *in vitro* raised plant material. The intensity of this transcript band varied according to the intensity of the actual transcript (900 bp) detected in the three genotypes but was less intense than the actual transcript. This could be another homologous transcript or the unprocessed primary transcript of PR1.

Fig. 4.2. Expression pattern of PR-1, test I.

Panels 1, 2, 3 and 4 represent the four replications while a, b, c and d represent the ethidium bromide stained northern gel, phosphorimager quantitative value for 25S (▲), autoradiograph after hybridisation with PR1 cDNA and phosphorimager quantitative value for the actual transcript (900 bp, □). Symbols ■ and ◁ represents the higher molecular weight transcript (~ 2.0 kb) and 18S rRNA band respectively. Replication 1,2,3 are the RNA samples from the plant material raised in the glasshouse while 4 represents the RNA samples from the *in vitro* raised plant material. The northern blot was washed for 10 minutes each with 6X SSC, 2X SSC and 0.1X SSPE at room temperature. Following these three washes another 10 minutes high stringency wash was given with 0.1X SSPE at 65°C. The northern blot was exposed for 36 hours to a maximum sensitivity X-ray Kodak film.

1a



2a



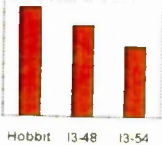
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4a



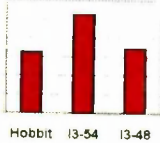
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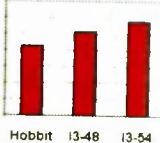
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3b



4b



1c



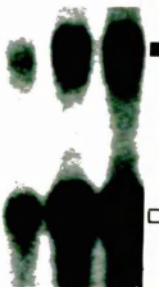
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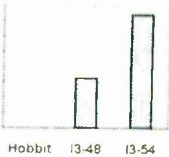
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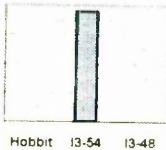
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2d



3d



4d

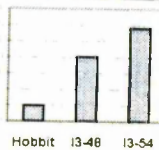


Table 4.4. Ratio of the expression value of PR-1 to 25S for three genotypes in four replications (three raised in glasshouse and one *in vitro*).

Genotypes	Replication 1	Replication 2	Replication 3	<i>in vitro</i>
<i>Hobbit sib</i>	-	-	-	0.58
<i>I3-48</i>	0.11	0.157	-	1.98
<i>I3-54</i>	0.34	0.13	0.14	2.52

Comparisons to be made within a replication not between replications.

4.2.2.3 Oxalate oxidase and peroxidase expression studies

A 904 bp barley cDNA, encoding oxalate oxidase, and a 1247 bp long cDNA from wheat encoding peroxidase were probe hybridised with two separate northern blots prepared similarly to the one used for PR1 expression studies. An induction of oxalate oxidase (OO) has been reported following inoculation with powdery mildew in cereals. It has been found to be strictly confined to the leaf mesophyll and might play a role in a signal transduction pathway for the regulation of resistance response mainly HR (Zhou *et al.*, 1998). Induction in expression of peroxidase (POX) gene has been reported to be associated with *E. graminis* resistance in wheat (Rebmann *et al.*, 1991b). Peroxidase might have some role in HR also as high levels of H₂O₂ have been reported to be synthesised in the cells undergoing or exhibiting HR. In the present study, no expression at mRNA level for these two PR proteins could be detected on the probed northern blots.

4.2.3. PR protein expression- test II

To check the consistency of the expression of TL and PR1, two northern blots were prepared from the RNA samples of the test-II plant material (raised with seedling test II). Total RNA of two mutants (I3-54 and I3-48) and Hobbit sib from three replications was loaded on the same gel. Alongside these nine samples, three RNA samples from the replication 1 of the previous test (designated here as replication 4) were also loaded. On probe hybridising one with TL (Fig. 4.3) and the

other with PR-1 (Fig. 4.4) cDNAs, similar results were observed to test I (Table. 4.5). The loading of RNA from the two mutants and Hobbit sib appeared approximately equal as judged by the intensity of rRNA ethidium bromide staining and the phosphoimager data (Fig. 4.3.panels a & b). The level of expression of the ~ 800 bp TL transcript was clearly higher in both I3-48 and I3-54 compared to Hobbit sib control in all the three replicates (Fig. 4.3 panels c & d). Similarly, the 900 bp PR1 transcript was detected at a higher level in both mutants overall three replicates (Fig. 4.4 panels c & d). However, the relative level of induction varied between replicates (Table 4.5). A weak higher molecular transcript for the northern blot probed with TL cDNA was also detected in all the three genotypes.

Table 4.5. Ratio of the expression value of TL and PR-1 to 25S for three genotypes in four replications (Test II).

Genotypes	Replication 1		Replication 2		Replication 3		Replication 4	
	TL	PR-1	TL	PR-1	TL	PR-1	TL	PR-1
<i>Hobbit sib</i>	-	-	-	-	-	-	-	-
<i>I3-48</i>	0.1	0.2	0.05	0.11	0.02	0.19	0.01	0.11
<i>I3-54</i>	0.07	0.18	0.04	0.21	0.07	0.30	0.03	0.22

Comparisons to be made within a replication not between replications.

4.5 Discussion

Expression of PR proteins has been reported to be associated with pathogen resistance response in plants. The production of these PR proteins is host specific and dependent upon its genetic constitution. Functions of most of these PR proteins are still unknown, but the majority of them have been reported to accumulate at a higher level in the resistant genotype during pathogen invasion (Davidson *et al.*, 1987, Davidson *et al.*, 1988, Boyd *et al.*, 1992, Bryngelsson *et al.*, 1994, Boyd *et al.*, 1995). There are also a few reports where PR proteins induction has been reported in some mutant genotypes grown under totally sterile conditions (Bowling *et al.*, 1994,

Fig. 4.3. Expression pattern of TL, test II.

Panels 1, 2, 3 and 4 represent the four replications while a, b, c and d represent the ethidium bromide stained northern gel, phosphorimager quantitative value for 25S (▲), autoradiograph after hybridisation with TL cDNA and phosphorimager quantitative value for the actual transcript (800 bp). Symbols ■ and ◁ represents the higher molecular weight transcript (~ 1.7 kb) and 18S rRNA band respectively. Replication 1,2,3 are the RNA samples from the plant material raised in the glasshouse test II while 4 represents the RNA samples from the replication 1, test I. The northern blot was washed for 10 minutes each with 6X SSC, 2X SSC and 0.1X SSPE at room temperature. Following these three washes another 10 minutes high stringency wash was given with 0.1X SSPE at 65°C. The northern blot was exposed for 48 hours to a maximum sensitivity X-ray Kodak film.

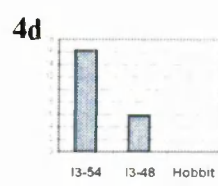
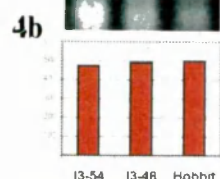
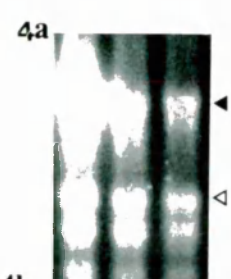
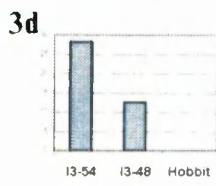
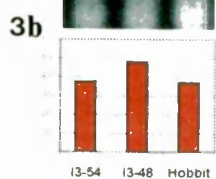
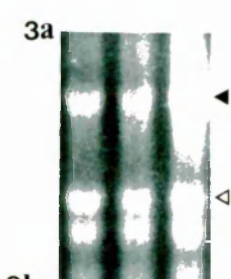
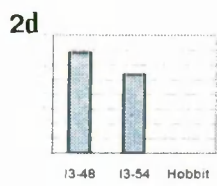
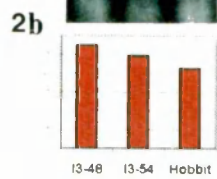
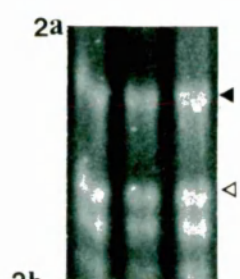
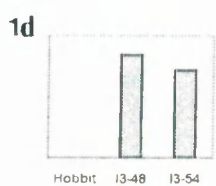
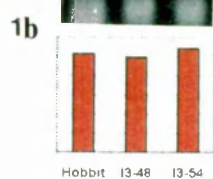
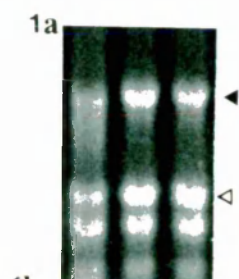
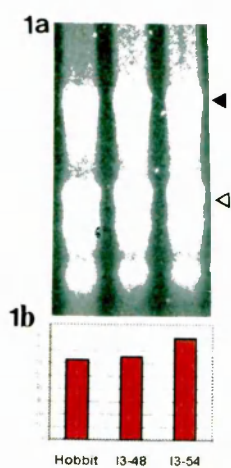
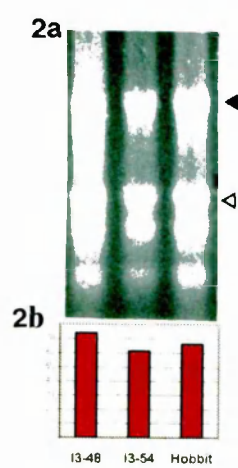


Fig. 4.4. Expression pattern of PR1, test II.

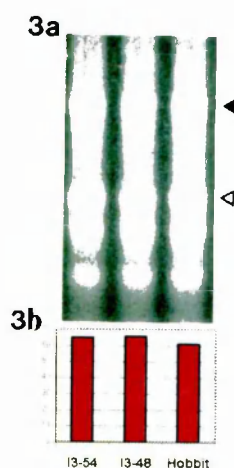
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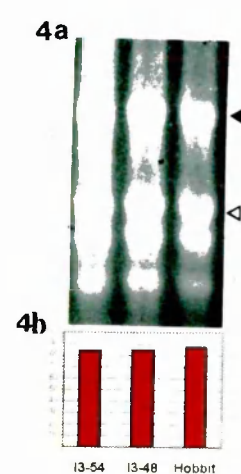
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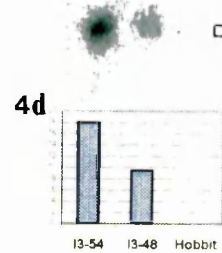
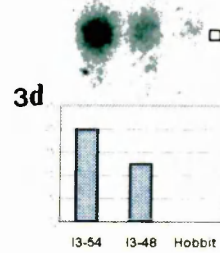
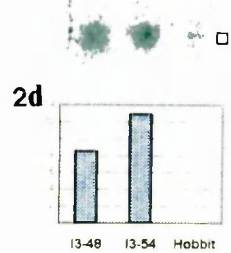
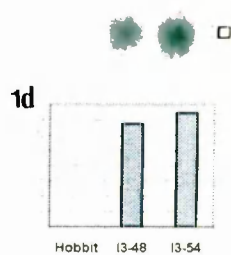
2c



3c



4c



Dietrich *et al.*, 1994). This suggests some developmental control of their induction. Mutants having constitutive expression of PR proteins were also found to be more resistant than the wild types and other mutants showing no induction of PR proteins. Similar results were observed in the present study on Hobbit sib deletion mutants. Higher levels of a TL and PR-1 mRNAs were present in the two mutants, I3-54 and I3-48 compared with the wild type 'Hobbit sib' control. These two mutants were also found to have significantly higher levels of mildew resistance at the third leaf stage, with I3-54 being the most resistant (Chapters 2 & 3). However no transcripts for OO and POX were detected. This suggests that the genes for these two enzymes might not be expressed constitutively in the resistant mutants or their expression levels were too low to be detected.

A comparison between the two mutants and the wild type 'Hobbit sib' clearly indicated that TL and PR-1 were expressed at a higher level in the two mutants in both independent tests. The expression of these genes might be regulated by the known and unknown deletions on these mutants (Worland and Law, 1991, Howie, 1997). PR proteins are often stress induced and in the present study, a strong induction of PR-1 was also observed in all the three genotypes raised *in vitro*. This could be due to restricted growing space and medium in the test tubes. However this induction was much lower in 'Hobbit sib' than in the two mutants. Also on the northern probe hybridised with TL a low level of transcript expression was detected for Hobbit sib in one of the replicates in test I and two replicates in test II. These plants might have been under some type of stress.

The level of expression of TL and PR1 in I3-48 and I3-54 was higher than detected in 'Hobbit sib' control in two independent experiments. However the extent

of induction appeared to vary between replications within an experiment. The reason for this variation was unclear but might relate to environmental interactions, which could have led to a further enhancement in some replicates.

A higher molecular weight transcript band was detected for TL in both the tests and PR-1 in the *in vitro* raised material. In case of TL, this transcript seemed to be present at approximately the same level in all the genotypes in test I but the expression levels were much lower in the test-II. Though similar stringency post hybridisation washes (time and salt concentration) were given to the northern in the two tests, the northern in test-II appeared to have received a higher stringency wash. A difference in the stringency of washing appeared to be the most likely explanation for the lower level of expression of the high molecular weight transcript in test II since replicate 4 in test II was actually a repeat loading of replicate I from test I. Overall this suggested the higher molecular weight transcript had less homology with the cDNA probe used.

In 1982 the 'Hobbit sib' hexaploid wheat genotype was irradiated and a series of deletion mutants having high levels of APR to yellow rust, brown rust and powdery mildew were identified (Worland and Law, 1991 and Howie, 1997). The mutations could have either led to the elimination of resistance gene suppressors or susceptibility genes from their hexaploid genomes. The present study was designed to characterise the powdery mildew resistance in the Hobbit sib deletion series by evaluating resistance at different plant developmental stages. Once that was established the fungal growth was compared between resistant mutants and the susceptible wild type. At the same time molecular changes due to mutation, which might have led to resistance were also determined.

5.1 Disease Evaluation

A series of powdery mildew disease tests on the Hobbit sib deletion series was conducted over two years to characterise resistance at different adult and seedling growth stages. I3-54 and I3-48 were observed to be significantly more resistant as both seedlings and adult plants than the wild type and the other five mutants. The resistance in these two mutants appeared to be under developmental control, expressing strongly from the third leaf stage of the seedlings. Wolters *et al* (1993) also observed that 14-21 days old seedlings of 'mlo' loci barley mutants were significantly more resistant than the 7 day old ones. They reported spontaneous development of some cell wall appositions in these resistant barley mutants even under disease free conditions. In a similar study done by Dietrich *et al* (1994) and Greenberg *et al* (1994) on *Arabidopsis* mutants, spontaneous formation of lesions resembling HR under disease free conditions were identified. In the present study, no such response

could be detected on I3-54 and I3-48, raised under disease free conditions. However leaves of I3-54 at the third leaf stage showed some level of chlorosis after inoculation. During resistance gene activation a whole array of products are synthesised in the host plant, which could accumulate to a higher level in the resistant genotype (Boyd *et al* 1992, Boyd *et al* 1995, Bryngelsson *et al* 1994, Davidson *et al* 1987 and Davidson *et al* 1988). A significant increase in some of these or their precursors could even be toxic to the plant (Baker *et al* 1998, Chen *et al* 1993, Draper, 1997, Peltomen *et al* 1998 and Wu *et al* 1997). So a higher induction of resistance gene associated compounds at the third leaf stage might occur in I3-54, leading to leaf chlorosis.

The resistance expressed in I3-48 and I3-54 appeared to be quantitative in nature, however the specificity could not be determined. The disease levels on these two mutants did not show a significant increase over 2-3 cycles of disease multiplication in the APR test and expression of resistance at different growth stages-I, which could be accounted for by selection of virulent pathotypes. However, to determine race specificity single spore isolates should be used for the initial inoculation under controlled environmental conditions.

5.2 Histological evaluation of mildew growth

The comparison of mildew growth between the wild type Hobbit sib and two resistant mutants indicated resistance to be expressed initially at the hyphal penetration stage. Once hyphae had penetrated the plant cell, haustorial development was slower in I3-54 than I3-48. Circumstantial evidence suggests that there is also a high level of post-haustorial resistance operative in I3-54 because of the low incidence of disease observed (Chapter.2) in comparison to the proportion of spores successful in producing haustoria. Also the third leaves of I3-54 showed some level of

necrosis/chlorosis after inoculation, which could be an indicator of post-haustorial resistance, associated with death of mesophyll cells leading to haustorial collapse or restricted growth (Boyd *et al*, 1995, Niks, 1983, Niks, 1991). This could have resulted in restricted sporulation and finally less disease on I3-54 as observed in various seedling tests (Chapter.2). However this hypothesis needs to be tested by quantifying colony size and sporulation. At the same time size of the haustoria and their functionality should be determined and subsequently related to the resistance levels.

In the histological studies done so far, no counts for papillae formation and HR cell death were made. The number of papillae with haloes on the two mutants and the wild type could be compared at 24 hai and cells undergoing HR by 48 hai for each genotype could be determined by observing the leaf segments under UV light. Plasmolysis and accumulation of neutral red dye could also be examined as an indicator of the viability of cells during pathogen infection. Lack of vital dye uptake and inability of cells to plasmolyse indicate loss of membrane's semi-permeability and cell death (Koga *et al.*, 1988).

5.3 PR proteins and resistance

To study the changes at the molecular levels due to mutation, which might have led to resistance in the mutants, the expression levels of various PR proteins were compared. The results from the molecular studies indicated high constitutive expression of two PR proteins; TL and PR-1, in I3-54 and I3-48's third leaves grown under non-inoculated conditions. This high constitutive expression might have contributed to resistance particularly to the pre-haustorial or penetration resistance without HR (Chapter.3) (Niks and Dekens, 1991). Yu *et al* (1998) suggested

constitutive induction of some systemic acquired resistance genes might substitute for HR cell death in potentiating stronger gene for gene defense responses.

On the other hand most of these PR proteins have been identified and isolated during the course of pathogen infection (resistance reaction). Also a biphasic pattern of accumulation in PR protein expression coinciding with PGT or AGT or haustorial formations has been reported by Davidson *et al* (1988), Clark *et al* (1993) and Boyd *et al* (1995). So after pathogen infection there might be a further induction in the expression level of TL and PR-1 and also a few more PR proteins associated with resistance could be detected. This higher expression could have led to high post-haustorial resistance or chlorosis especially in I3-54.

A limited study was also carried out to determine the constitutive expression of PR proteins in the second leaf stage of the seedlings (more susceptible than the third leaf stage seedlings, Chapter 2) also. The results indicated lower expression levels of TL in I3-54 and I3-48 at second leaf stage in comparison with the third leaf stage. Whilst for PR-1 the expression levels were too low to be detected. The results from the *in vitro* raised seedlings showed a higher induction even in the wild type for PR1. So these *in vitro* seedlings could be tested for mildew growth and compared with the PR protein induction levels. This would help in determining the association of PR protein induction with resistance more closely.

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